

# **Epidemiology of Ovine Paratuberculosis in New Zealand**

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## Abstract

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The overall goal of this PhD project was to better understand the general epidemiology of ovine paratuberculosis (PTb) and the specific molecular characteristics of the causing organism *Mycobacterium avium* subspecies *paratuberculosis* in New Zealand.

To begin with, current control measures for clinical PTb in New Zealand's major pastoral livestock species (dairy cattle, beef cattle, sheep, deer) were reviewed. Infection with *Map* is common in all these species and control is voluntary for all livestock industries. Control measures aim to reduce the incidence rate of clinical PTb rather than to eradicate *Map* infection. Dairy and deer industries have developed resources describing best-practice management options that assist farmers and veterinarians to advise their clients about specific control plans. There is no national control programme for sheep and beef cattle. However, unlike for cattle and deer, the use of a commercial vaccine is licensed for sheep. Evidence in this thesis suggests that vaccination may be a cost effective option for flocks that experience a high incidence of clinical disease. For deer, there is a national abattoir surveillance programme that aims to alert farmers of unusually high rates of PTb-like lesions in deer at slaughter. Evaluations of the biological and economic effectiveness of voluntary control still remains to be undertaken for all industries.

Work in this thesis estimated the on-farm economic cost of clinical PTb in sheep (ovine Johne's disease, OJD) in New Zealand. It was based on data about the incidence of clinical PTb and overall mortality from 20 OJD-affected farms. The benefit-cost ratio of vaccination was estimated. Farms were categorized as either fine-wool breed (Merino, Half-bred, Corriedale) or other breeds and calculations were stratified for these two farm categories. The estimated mortality due to OJD was 2.7 times as high in fine-wool as in other breeds with large variation between farms. A stochastic simulation for a hypothetical flock with 2,000 breeding ewes resulted in an average annual cost of OJD-mortality of NZ \$13,100 in fine-wool and NZ \$4,300 in other breeds. Vaccinating replacement lambs

against OJD would be cost-effective in most flocks when the pre-vaccination annual OJD ewe mortality was  $>1\%$ . Accurate on-farm observation of OJD to establish incidence would help farmers to make better decisions about vaccination.

Frozen-stored faecal and serum samples of individual sheep with no signs of clinical disease from 45 commercial flocks from a 2013 study that determined pooled faecal culture (PFC) status were used to determine faecal *Map* shedding and antibody in serum. A total of 878 faecal samples were tested with direct faecal real-time quantitative PCR (qPCR) to determine *Map* shedding prevalence and abundance in individual animals. In addition, the qPCR results were compared with *Map* antibody ELISA results from 837 corresponding sera to correlate the observed shedding prevalence with sero-prevalence. Overall, 13.1% of faecal samples and 5.8% of serum samples tested positive. The median intra-flock prevalence (IFP) of *Map* shedding in the qPCR positive flocks was 13.5% with a range of 5–95%. The median IFP of *Map* ELISA antibody positive flocks was 10% with a range of 5–20%. ELISA results and the DNA concentration in qPCR positive samples were positively correlated. Nevertheless, ELISA was a poor predictor of individual shedding. A more robust assessment of the shedding status of flocks can be achieved by using a combination of qPCR and ELISA of individual animals rather than a single PFC of 20 randomly selected sheep per flock, as was used in the 2013 survey.

Type S1 *Map* isolates from New Zealand and the Australian Telford strain were characterized based on single nucleotide variant (SNV) analysis of whole genome sequence data (WGS). A Type S1 genome was completely sequenced and closed for using as a reference for the SNV analysis. Besides defining the genetic relationship between *Map* isolates from New Zealand, Australia and Europe several phenotypic variables used as surrogates for the severity of PTb in individual hosts were investigated. The New Zealand and Australian isolates formed a closely related group. They were distinctly different from the Type S isolates from countries in Europe. Within New Zealand, *Map* genotypes and region of sheep farm locations were significantly associated ( $p < 0.05$ ). There were no significant associations between genotype and surrogates for severity, observed in the animals which the genotypes were isolated from, such as histopathological scores of intestinal lesions, host serology or the gross-pathological diagnosis by veterinarians at necropsy. These results suggested that the phenotypic variation of PTb may depend on factors other than *Map*-genotype in Type S strain. Further studies are required to sub-

stantiate a hypothesis about varying virulence factors of the *Map* genome in New Zealand sheep.

In summary, PTb control in New Zealand is voluntary in all major ruminant livestock industries including sheep. In clinically affected commercial sheep farms, estimated mortality due to OJD was 2.7 times as high in fine-wool as in other breeds, but large variations were observed between farms. PTb vaccination in commercial sheep flocks may be cost effective if annual incidence of OJD attributable ewe mortality is  $>1\%$ . In non-clinical commercial flocks, the median IFP of *Map* shedding and *Map* ELISA antibody positive prevalence was 13.5% and 10% respectively. Approximately 1% ewes in qPCR positive flocks were supershedders. Based on analysis of WGS data, Type S *Map* genotypes from New Zealand sheep were similar across the country and not affected by the type of breed or disease outcome in hosts.



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## **Nomenclature**

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AFB	Acid fast bacillus
AGID	Agar gel immunodiffusion
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
BWA	Burrows-wheeler aligner
BCS	Body condition score
CI	Confidence interval
CTAB	Cetyl trimethyl ammonium bromide
CTC	Cetylpyridinium chloride
DNA	Deoxyribonucleic acid
DNZ	Dairy New Zealand
ELISA	Enzyme-linked immunosorbent assay
EU	ELISA unit
IFN $\gamma$	Interferon gamma
IFP	Intra-flock prevalence
IQR	Interquartile range
IS	Insertion sequence
JD	Johne's disease
JDRC	Johne's Disease Research Consortium
<i>Map</i>	<i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i>
MS	Microsoft
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NZ	New Zealand
NZGL	New Zealand Genomics Limited
OJD	Ovine Johne's disease
PFC	Pooled faecal culture
PhD	Doctorate of Philosophy
PTb	Paratuberculosis
qPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
S/P	Sample to positive
SCHALS	Subcommittee on Animal Health Laboratory Standards
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SSR	Simple sequence repeat
UK	United Kingdom
USA	United States of America
VNTR	Variable number tandem repeat
WGS	Whole genome sequencing

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## List of Publications

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Gautam, M., Ridler, A., Wilson, P.R., Heuer, H., (2018) Control of clinical paratuberculosis in New Zealand pastoral livestock. *New Zealand Veterinary Journal* **66 (1)**, 1 – 8. doi: 10.1080/00480169.2017.1379914

Gautam, M., Anderson, P., Ridler, A., Wilson, P., Heuer, H. (2018) Economic cost of ovine Johne's disease in clinically affected New Zealand flocks and benefit-cost of vaccination. *Veterinary Sciences* **5(16)**, 1 – 13. doi:10.3390/vetsci5010016

Brauning, R., Plain, K., Gautam, M., Russel, T., Correa, C.C., Biggs, P., Whittington, R., Murray, A., Price-Carter, M. (2019) Complete genome sequence of the Telford type S strain of *Mycobacterium avium* subsp. *paratuberculosis*. *Microbiology Resource Announcement*. (**Accepted for publication Feb 2019** ). doi:10.1128/MRA.00004-19



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## General introduction

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Paratuberculosis (PTb) is a bacterial infection caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). Two major types of *Map* exist: Type S and C. While Type S was originally isolated from sheep and Type C from cattle—hence the naming—*Map* is not host specific (Whittington et al. 2017). Phylogenetic analysis using modern methods such as whole genome sequencing (WGS) have identified two subtypes of Type S (subtype I and III) and Type C (subtype B and subtype C). Type C subtype B further comprises the Indian bison and USA bison sub-subtypes (Fawzy et al. 2018). Subtype I and subtype III of the Type S have been referred to as Type S1 and S3 respectively throughout the rest of this thesis. While infection with *Map* mostly remains subclinical, the clinical disease expresses as chronic enteritis leading to death of the affected animal. The clinical form of *Map* infection is called Johne's disease (JD). While PTb and Johne's disease (JD) are terms that are often used interchangeably, in this thesis the latter has been used only to refer to the clinical disease. Additionally, the term clinical PTb has been used as a synonym for JD.

Massey University is one of the leading veterinary/agriculture research organisations within New Zealand that has undertaken PTb research studies. Five previous PhD programmes (Norton 2007, Stringer 2010, Hunnam 2011, Verdugo 2013, Marquetoux 2017) completed at the School of Veterinary Science (formerly the Institute of Veterinary, Animal and Biomedical Sciences) at Massey University have investigated aspects of its epidemiology among dairy cattle, sheep, beef cattle and deer. They reviewed current knowledge, surveyed the prevalence of infected herds and flocks, clinical disease incidence, described the distribution of genetic strain types of *Map* and their association with species and location, evaluated transmission between species and between farms through animal

movements, estimated farm-level economic effects, and developed mathematical models to compare the relative merit of various measures of disease control. The present thesis focuses on ovine PTb in New Zealand and builds and expands on the knowledge from the previous work at Massey University in order to investigate some new hypotheses. The overall goal of this PhD project was to understand epidemiology and microbiology of PTb and the specific molecular characteristics of the causing organism, *Map*, with a focus on ovine species in New Zealand in a more comprehensive manner than reported previously.

## 1.1 Structure of the thesis

This thesis includes four independent research chapters intended for peer-reviewed publication and a general discussion as a standalone chapter. Formatting chapters as independent manuscripts for publication necessarily entailed some repetition of text in the introduction sections. While research chapters are interrelated in terms of samples collected or used for data generation and contributed equally to the overall goal of the project, each independent chapter has a different focus, which can be broadly categorised as follows:

1. review of clinical PTb control programs in New Zealand;
2. economic analysis of vaccination as a control method in affected flocks;
3. diagnosis of *Map* shedding and exposure at an individual animal level;
4. molecular analyses of *Map* genomes based on whole genome sequencing using the Telford1 (National Center for Biotechnology Information (NCBI) accession number CP033688) reference strain of *Map*.

The general discussion chapter, which follows the independent research chapters, encompasses major findings and limitations of these independent chapters thereby highlighting how these chapters are interrelated. Overall findings presented in this thesis help to understand various epidemiological features of *Map* and ovine PTb in New Zealand. The information may be used to prioritize measures for ovine PTb control and to facilitate future research in pastoral farming systems.

## 1.2 Research chapters

### 1.2.1 Review of clinical PTb control programs in New Zealand

The first research chapter (Chapter 2) is a review of measures to control clinical PTb in major New Zealand pastoral livestock species namely dairy cattle, beef cattle, sheep and deer. Previous doctoral theses completed at Massey University have reviewed the general epidemiology of clinical and non-clinical PTb along with the spatial and species distribution of molecular *Map* strains. Additionally, diagnosis and pathogenesis of the disease have been reviewed. Hence, in order to avoid duplication the focus was on review of clinical PTb occurrence and current control practices in New Zealand pastoral species. Additional aspects such as economics of vaccination for PTb control, diagnosis of *Map* shedding and exposure and molecular epidemiology of *Map* are subjects of separate research chapters (3-5).

In order to co-ordinate PTb research in New Zealand, a joint partnership program called the Johne's Disease Research Consortium (JDRC) was funded by livestock industries, and Government from 2008-2016 and implemented by researchers of various institutions. The goal of the JDRC programme was to develop practical and cost effective management tools in order to help reduce the overall prevalence of PTb in New Zealand livestock flocks or herds. A review of PTb control in New Zealand was therefore timely. A review of bovine PTb control activities in endemically infected developed countries (Geraghty et al. 2014) did not include any information about New Zealand. Such reviews not only serve as a convenient source of useful information about background, control activities and monitoring components, they also demonstrate a country's disease control efforts to consumers. The public perception about *Map* might be of special interest for consumers of meat and milk products due to the purported zoonotic association between *Map* and Crohn's disease. Therefore, easily accessible, systematic information about disease control measures in New Zealand may facilitate continued access to international markets for livestock and food products. Hence, the review will serve as a baseline source of information for anybody interested in PTb in New Zealand and ongoing PTb control activities in New Zealand.

### 1.2.2 Economic analysis of vaccination as a control method in affected flocks

In Chapter 3, estimates of mortality due to clinical PTb and associated cost in affected commercial sheep flocks were obtained and then these parameters were used to undertake simulation-based benefit-cost analysis of vaccination as a control method. Ovine Johne's disease (OJD)-specific mortality data from 15 fine-wool breed (Merino and Merino X and Half-Bred) and five sheep breeding and finishing farms with other breeds (Romney and Composite breeds) were analysed. All farms had evidence of clinical OJD. While *Map* infection is reported to be widespread in New Zealand's sheep farms with an estimated national flock prevalence of 76% (Verdugo et al. 2014a), a national program to monitor ovine PTb does not exist and a well-researched current assessment of the economic cost of OJD or *Map* infection at the farm level is lacking, justifying research presented in this chapter.

Studies about economic impact of ovine PTb in sheep flocks are limited. In dairy cows, both subclinical and clinical forms of PTb are recognized as a production limiting diseases (de Lisle & Milestone 1989, Hutchinson 1996, Norton 2007). In New Zealand, economic impact of ovine PTb is arguably less relative to other health conditions such as internal parasitism or facial eczema although reliable information on the economic cost of the former is not available. Sheep with clinical PTb have been reported to have lesser live body and fleece weights and a fewer number of lambs born per ewe per year compared to healthy ewes (Morris et al. 2006). Given that the majority of *Map* infected animals are infected subclinically and the currently available ante-mortem tests can only imperfectly diagnose *Map* infection in such animals, it is difficult to assess the distribution and impact of subclinical disease in individual sheep. Consequently, estimates of physically monitored production effects of subclinical PTb in sheep are unavailable in New Zealand. Based on a non-peer reviewed simulation study published in 1998, the minimum annual cost of OJD in New Zealand was NZ \$0.9 million when 6% of the flocks in the country were presumed to be infected (Brett 1998). However, if 70% of flocks were infected (Verdugo et al. 2014a), the estimated annual loss would increase to NZ \$9.9 million from 0.9 million (Brett 1998).

Data collected and analysed in Chapter 3 helped us to estimate OJD-related mortality in

affected flocks as well as thresholds of OJD incidence in infected flocks above which control becomes financially attractive for farmers. In addition, these data were used to parameterise mathematical models to estimate total production loss due to OJD.

### 1.2.3 Diagnosis of *Map* shedding and exposure at an individual animal level

In Chapter 4, a subset of a large repository of frozen sheep faecal and serum samples from a previous national survey of commercial New Zealand sheep flocks (Verdugo et al. 2014a) was tested to estimate intra-flock prevalence (IFP) of *Map* shedding and serum antibody ELISA positivity (*Map* exposure) in New Zealand. Faecal samples of adult ewes without clinical signs were tested by quantitative PCR (qPCR) and the corresponding serum samples by commercial ELISA. The focus was on the prevalence of multi-bacillary animals that were possible major contributors to within-flock infection dynamics and that may be detectable by common diagnostic tests such as PCR and/or ELISA. In cattle, the term super shedders was used for high-shedding animals in a herd, which were postulated to act as major source of ongoing environmental contamination in the farm (Pradhan et al. 2011, Aly et al. 2012). It would be biologically plausible that the identification and elimination of these high shedding animals, that disproportionately contaminate the farm environment, can be an effective way to reduce new infections and the risk of clinical disease while minimising the number of culls for biological reasons and thereby reducing the cost of disease control (Heuer et al. 2011).

Prevalence of PTb is often difficult to estimate reliably as current ante-mortem diagnostic methods are not perfect. Under natural conditions clinical PTb has an unusually long incubation period that can range up to a few years (Chiodini et al. 1984). Usually in sheep the clinical disease is seen in animals older than two years (Whittington & Sergeant 2001). Since the infection with *Map per se* is highly insidious, test methods often have limited sensitivity to reliably estimate the true prevalence of infection, particularly at an early stage (Magombedze et al. 2013) and until the infected host exhibits clinical signs. Published reports about the animal prevalence of *Map* shedding and exposure within infected New Zealand sheep flocks was lacking. Chapter 4 therefore estimates the IFP of *Map* shedding and exposure in individual animals in commercial sheep flocks in New

Zealand.

#### **1.2.4 Molecular analyses of *Map* genomes based on whole genome sequencing using the Telford1 reference strain of *Map***

In Chapter 5 whole genome sequences of several *Map* isolates collected from ewes with different histopathological scores of intestinal lesions due to *Map* infection described by Prez et al. (1996) were compared to the closed genome, Telford1, a Type S1 *Map* genome, described in Appendix 1. A deeper understanding of the various aspects of *Map* infection and the disease dynamics will be useful for developing effective prevention or control strategies.

Several previous research reports have suggested that *Map* subtypes have different infectivity and/or pathogenesis for different host species (OBrien et al. 2006, Verna et al. 2007, Fernandez et al. 2014). New Zealand's mixed species pastoral farming system is conducive to close contact between animals and transmission of *Map* between co-grazed species. Traditional genotyping methods such as variable number tandem repeats (VNTR) and short sequence repeats (SSR), which rely on *Map*-specific interspersed repetitive units of nucleotide sequences for genetic characterization, have been helpful for identifying the host preference of *Map* subtypes and for demonstrating the tendency of co-grazed animals to share the same subtypes (Verdugo et al. 2014a). However, these assays have only a limited ability to distinguish different Type S *Map* isolates.

Whole genome sequencing (WGS) has greatly enhanced the ability to distinguish Type C *Map* isolates (Ahlstrom et al. 2015, Stevenson 2015). It seems likely that this technology will also enhance the ability to further differentiate Type S *Map* and potentially provide a means to investigate different virulence properties of *Map* and/or facilitate the back/forward tracing of infection chains (Bannantine et al. 2013). The identification of more or less virulent subtypes, if they exist, may reveal immunogenic properties of less virulent subtypes that can be harnessed to protect susceptible hosts from the effects of highly virulent strains. In chapter 5 of this thesis, whole genome sequence data of 69 Type S1 *Map* isolates from New Zealand and Australia were analysed for identification of point mutations of single nucleotides called SNVs (single nucleotide variants). We then compared how these variations at the genetic level correlated to the phenotypic ex-

pression of disease as measured by the histological scores of the lesions, serology status and provisional diagnosis by vets based on necropsy.

The thesis concludes with a general discussion about the collective knowledge gained from individual studies and how this informs the current presentation of PTb and its control, as well as future research. Based on overall findings and exposure of knowledge deficits by this thesis, ideas/areas for future research are proposed.



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## Control of clinical paratuberculosis in New Zealand pastoral livestock

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**Abstract** – This review summarises current control measures for clinical paratuberculosis (Johne’s disease; JD) in New Zealand pastoral livestock. Most New Zealand sheep, deer, beef and dairy cattle herds and flocks are infected by *Mycobacterium avium* ssp. *paratuberculosis* (*Map*). Dairy cattle and deer are mostly infected with bovine (Type C), and sheep and beef cattle with ovine (Type S) strains. Control in all industries is voluntary. While control in sheep and beef cattle is *ad hoc*, the dairy and deer industries have developed resources to assist development of farm-specific programmes.

The primary target for all livestock is reduction of the incidence rate of clinical disease rather than bacterial eradication *per se*. For dairy farms, a nationally instituted JD-specific programme provides guidelines for risk management, monitoring and testing clinically suspect animals. While there is no formal programme for sheep farms, for those with annual prevalences of clinical disease >2%, especially fine-wool breeds, vaccination may be a cost-effective control option. The deer industry proactively monitors infection by a national abattoir surveillance programme and farmers with an apparent high disease incidence are encouraged to engage with a national network of trained consultants for management and control advice. Evaluation of the biological and economic effectiveness of control in all industries remains to be undertaken. Nevertheless, opportunities exist for farmers who perceive significant JD problems in their herds/flocks to participate in systematic best-practice activities that are likely to reduce the number of clinical infections with *Map* on their farms and therefore the overall prevalence of JD in New Zealand’s farming industries.

**Key words:** Johne’s disease, *Mycobacterium avium* subspecies *paratuberculosis* (*Map*), control, sheep, cattle, deer, New Zealand

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### 2.1 Introduction

Paratuberculosis (PTb) is a globally endemic, chronic, contagious bacterial infection of cattle, sheep, goats, deer and wild ruminants that can cause clinical disease characterised

by a long incubation period, progressive wasting and death (Harris & Barletta 2001, Sweeney et al. 2012). It is caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*), which has two major strain types; Type I which was historically known as Type S as it was first isolated from sheep and Type II, historically known as Type C owing to its first culture from cattle (Stevenson 2015). A subtype of Type S named Type III also exists (Stevenson 2015). Infection with *Map* generally remains subclinical. In this review Johne's disease (JD) refers specifically to the clinical manifestation of PTb, whereas infection with *Map*, includes subclinical and clinical infections. Imperfect sensitivity and specificity of diagnostic tests for early or subclinical infections remains a limitation for diagnosis and control. Hence, herd infection status is a more appropriate diagnostic measure than that of an individual animal. Eradication of *Map* is challenging and likely unachievable, therefore the aim of control measures is to minimise exposure and consequently clinical disease incidence.

In New Zealand, PTb is endemic in farmed cattle, sheep and deer (Table 2.1), but until recently no nationally coordinated control programme has existed for any species. However, control programmes initiated by New Zealand's international trade competitors motivated livestock industries to address control more seriously (Burton & Voges 2002). From 2008-2016, the Johne's Disease Research Consortium (JDRC), a partnership between livestock industries, researchers and the Government undertook a coordinated programme to develop practical and cost-efficient management tools. A review of PTb control in New Zealand is therefore timely. Control elsewhere has been reviewed by Geraghty et al. (2014). This review focuses on control activities in New Zealand, but it draws upon relevant literature from elsewhere.

Information sources used in this review include published material available electronically and manually. Peer-reviewed sources on many aspects of PTb control specific to New Zealand are limited, hence it was unavoidable to include literature from non-reviewed sources. Only sources considered by the authors to be robust have been included and they are referenced appropriately. The objective of this review is to outline key aspects of the control of clinical PTb in dairy cattle, sheep and farmed deer in New Zealand.

## 2.2 Cattle

### 2.2.1 Epidemiology

In New Zealand dairy cattle, infection with *Map* is mainly associated with Type C, but sporadically Type S (Verdugo et al. 2014b). Recent cross-sectional studies estimated 47–54% of dairy herds were infected (Norton et al. 2009, Hunnam 2014), although those studies were based on farmer diagnosis, which would have affected sensitivity and specificity of diagnosis.

In beef cattle, Verdugo et al. (2014a) reported herd-level prevalence of 42% based on faecal culture or serum ELISA, with 80% being Type S, likely attributable to co-grazing with sheep. Verdugo (2013) reported the mean annual incidence of JD was 0.21% on infected beef farms, based on farmer diagnosis. Given the low incidence and the absence of literature on JD control in beef cattle, we focus largely on dairy cattle.

### 2.2.2 Diagnosis

Current ante-mortem diagnostic tests commonly used in individual animals are faecal culture and faecal PCR assays that detect *Map* shedding, or tests of serum or milk for host immune response against *Map* (Table 2.2). While direct measures of *Map* shedding are provided by faecal tests, they are unable to differentiate between active shedding due to true infection and passive shedding occurring as a result of mere ingestion (Whittington et al. 2019). ELISA to detect *Map* specific antibodies in serum or milk are less expensive, easier to use and have a shorter turnaround time than faecal culture or PCR assays. Less commonly used tests to detect humoral immune responses include agar gel immunodiffusion (AGID) and complement fixation test (CFT) (Whittington et al. 2019). These ante-mortem tests for humoral immunity in general have low sensitivity in latent and sub-clinically infected animals. However, clinically affected animals shed large numbers of *Map* in faeces and their antibody titres are usually high, hence tests perform reasonably well in that class of animals (Nielsen 2014). Current commercially available ELISA for cattle have a specificity of >98% (Nielsen 2014). Using test results from individual animals in the context of the herd history will provide a better understanding of the the

disease status in a herd (Whittington et al. 2019).

Lack of a gold standard test and variation in sensitivity depending upon the stage of infection in individual animals poses challenges in test evaluation. Norton et al. (2010) retested cows in four dairy herds with a history of clinical disease, using serum ELISA and faecal culture, over three lactations. Using the Bayesian regression technique to estimate the performance of these tests, in the absence of a gold standard, they found that ELISA, but not faecal culture, was more sensitive in older animals. Sensitivity of ELISA and faecal culture were 28 and 18% higher, respectively, in early lactation compared to late lactation. This was possibly due to physiological stress associated with calving and peak milk production in early lactation resulting in altered immunological response to *Map* infection or activation of latent infections.

In naturally infected dairy cows in Denmark, of cows that were categorised based on faecal culture as intermittent, low or high *Map* shedders, 30, 60 and 70%, respectively, tested positive for *Map* antibodies by milk ELISA at the time they first tested positive by faecal culture (Nielsen 2008). This observation seroconversion preceding bacterial shedding suggests that ELISA might be used to diagnose *Map*-infected animals before they become infectious.

Milk and serum ELISA generally have a similar range in animal-level sensitivities; hence ease of milk collection makes the milk ELISA a cost-effective option for herd screening (Nielsen 2014). In a review of ante-mortem diagnostic test for detection of *Map* infection, sensitivity and specificity of milk ELISA in cows that shed *Map* at the time of testing were reported to range from 21–61% and 83–100%, respectively, compared with sensitivities of 24–94% and specificities of 41–100% for serum ELISA (Nielsen & Toft 2008).

For diagnosis of *Map* infection at the herd level, culture or PCR on pooled faeces to detect *Map* organisms, or ELISA for bulk tank milk to detect antibodies to *Map* can be used. The advantages and disadvantages of using these techniques have been reviewed by (Nielsen 2014). Due to the relatively modest cost and time involved, the JDRC recommended primary herd testing by ELISA followed by optional PCR testing for confirmation, for routine herd screening in New Zealand (Anonymous 2015a). With modifications, this approach was also used in Australia, Canada, Denmark, the Netherlands, the United Kingdom and the United States of America for surveillance (Geraghty et al. 2014).

The diagnostic test employed depends on the cost-benefit and purpose of the control programme. Hence one test strategy does not fit all situations. While herd-level testing can provide a useful guide in estimating infection prevalence, cow-level tests are required to identify infectious animals (Nielsen 2014).

### 2.2.3 Control

In New Zealand, JD became notifiable in the 1930s, but was later delisted due to the lack of adequate tests or adoption of advised farming practices (de Lisle 2002). Hence control is not compulsory (Table 2.3). In 2015, DairyNZ (Hamilton, New Zealand), via JDRC, produced a guideline manual for the management of JD in dairy herds (Anonymous 2015b). The primary goal was reduction of the number of clinical cases and production losses rather than eradication of JD, by reducing exposure of young stock to infection. Prevention of infection in newborn calves and young stock is one of the key strategies because calves, in their first year of life, are significantly more susceptible to infection with *Map* compared to adult cows (Windsor & Whittington 2010). Other factors that have been identified as increasing the risk of infection include breed, with Jersey cattle being more susceptible, use of paddocks which have been used for grazing sick animals for grazing calves, and unrestricted animal and herd movements (Jakobsen et al. 2000, Norton et al. 2009, Marquetoux et al. 2016).

The key management strategies include the following.

#### *Determination of infection status*

Veterinary assistance is recommended to assess the infection status of a farm using clinical records, whole-herd milk testing using ELISA, testing older and/or culled poor condition cows using serum ELISA and/or faecal PCR assays. Once infection is diagnosed, a tailored plan should be developed with veterinary consultation, as determined from an inventory of key on-farm risk practices.

#### *Test and cull high risk cattle*

The aim is to reduce the likely major source of exposure of young stock to *Map*. This is achieved by testing clinically suspect cows by serum ELISA, and testing all individual

milk samples annually using ELISA, followed by immediate culling of high-positive cows and their calves, along with calves of clinically diseased cows. Alternatively, test-positive cows may be retained until the end of the season for economic reasons, but they should be isolated and calved in separate paddocks. Testing using ELISA can detect pre-clinical animals with high titres indicating they are likely shedders, thus removing them early would reduce contamination.

It must be noted that cows with clinical signs represent only a small fraction of infected cows in a herd and shedding starts, albeit in low numbers, even before clinical signs appear (Whitlock & Buergelt 1996). Furthermore, delays in culling infected cows may perpetuate exposure and in poorly managed herds more than one annual test may be required (Lu et al. 2008). However, in a survey of dairy farms investigating management of cows diagnosed or suspected of having JD, fewer than half the farmers followed the recommendation to cull within a week and less than a quarter followed the recommendation to graze infected cows separately on pasture blocks before culling. Most used risky practices such as grazing them in hospital paddocks or leaving them in the milking herd (Hunnam 2014).

#### *Calving and colostrum management*

This aims to reduce the risk of transmission of *Map* to calves by early removal from dams, feeding low risk colostrum from cows that repeatedly test negative on screening tests, retention of calves only from these test-negative cows, providing clean drinking water and feeding clean pasture to calved cows, preventing young stock from contact with effluent, using milk replacer for replacement calves and monitoring the herd for early clinical signs of disease. Alternative options are to not rear calves from cows that tested positive on ELISA (high-risk cows), feed colostrum only from 2–4-year-old-cows that tested negative and hence are low risk, and keep calves separate from adult cows and their pasture.

Currently, the immediate removal of calves after birth is uncommon on New Zealand dairy farms, with most removing them after 12–24 hours (Norton et al. 2009). Any delay in removal of calves potentially allows them to consume infected colostrum. Although feeding pooled colostrum is considered a risky practice (Nielsen et al. 2008), fewer than 3% of dairy farmers reported feeding pooled colostrum from low risk cows, while most used pooled colostrum from any cows indiscriminately (Hunnam 2014).

*Replacement heifer management*

This aims to reduce the risk of infection in replacement heifers by using a dedicated grazing area immediately after weaning, preventing exposure to effluent and provision of clean drinking water. Alternatives include grazing replacement heifers and adult cows separately, not grazing pasture for at least three months after adult cows, and grazing away from the milking platform.

*Biosecurity*

This aims to avoid introducing *Map* from high-risk sources. Limited co-grazing of dairy cows with other species minimises the risk of cross-infection (Verdugo 2013). If a closed herd status cannot be maintained, it is recommended to test cows and bulls before introduction, verify history of the herd of origin and ensure intact boundary fencing. Alternative interventions include buying only young cows, isolating and testing new additions and avoid grazing of adult cows on heifer rearing blocks. Nevertheless, 90% of farmers purchasing cattle stated they were unaware of the JD status or history of the source farm, indicating a lack of vigilance (Hunnam 2014).

Even though most dairy farms are infected, the number with high incidence of JD and severe production loss appears small. Consequently, a compulsory national control programme for the dairy industry does not appear to be warranted unless zoonotic risk is established and food safety becomes an imperative. Management is considered the most effective tool for control of JD (Harris & Barletta 2001).

Field trials and simulation studies on vaccination and genetic selection for control of *Map* infection are available from overseas. Vaccines limit spread by reducing faecal shedding and incidence of clinical disease, but do not eliminate infection (Knust et al. 2013). In New Zealand, an oil-adjuvant vaccine (killed *Map* 316F strain; Silirum<sup>TM</sup>; Zoetis), is licensed for cattle, but is not marketed due to possible interference with tuberculosis testing (Clough et al. 2010). This restriction is unlikely to change in the short-term as priority is given to achieve tuberculosis-free status in livestock by 2026. Genetic selection for resistance is deemed to have limited practical scope due to the long lead-time for elimination of *Map* infection (van Hulzen et al. 2014).

## 2.3 Sheep

### 2.3.1 Epidemiology

Infection with *Map*, usually Type S, is widespread in New Zealand sheep with an estimated flock-level prevalence of 76% (Verdugo et al. 2014a). Reported average annual JD incidence in infected flocks was <1% (Verdugo (2013); Table 2.1). By contrast, in Australia, the flock-level prevalence was reported to be 2.4–4.4%, mainly clustered in New South Wales (Sergeant & Baldock 2002). Annual incidence of JD ranged from 2–18% (Bush et al. 2006).

### 2.3.2 Diagnosis

Unlike in cattle, profuse diarrhoea is not common in clinically affected sheep. Accurate diagnosis requires laboratory diagnostic testing (Robbe-Austerman 2011). As sheep and goats in very poor body condition bear little or no economic value, clinical disease can be confirmed by on-farm necropsy (Robbe-Austerman (2011); Table 2.2). Routine serological testing of individual animals without clinical signs is not cost-effective although testing may be useful when targeted at adult animals with poor body condition.

For flock-level detection, pooled faecal culture (PFC) can be used cost-effectively to diagnose presence of *Map* (Whittington et al. 2000a). Flock-level sensitivities of PFC and serology (AGID) were estimated in Australian flocks with high ( $\geq 2\%$ ) and low ( $< 2\%$ ) prevalence of infection, with infection status confirmed by post-mortem and histological examinations (Sergeant et al. 2002). PFC had higher sensitivity (92 (95% CI = 82.4–94.7%)) than AGID (61 (95% CI = 50.5–70.9%)). Flock-level sensitivity of PFC was reported to be influenced by proportion of multibacillary cases, the pool size and disease prevalence (Dhand et al. 2010).

The sheep strain of *Map* does not grow well in traditional artificial solid media (Whittington et al. 1999), but has been successfully cultured using liquid culture media. The liquid media system is more expensive than solid media as cultures need to be confirmed by Ziehl-Neelsen staining or PCR (Whittington 2009).

Antibody-based serological tests such as ELISA or AGID have limited sensitivity, even in animals at advanced stages of disease, due to individual variability in immune responses (Whittington & Sergeant 2001). The diagnostic sensitivity of ELISA and AGID tests, using histopathology or culture as the gold standards to confirm infection in sheep, were 35 (95% CI = 27.3-43)% and 14 (95% CI = 8.8-20.3)%, respectively. Sensitivity of both tests varied in relation to the extent of histological lesions (Gumber et al. 2006).

A PCR assay for detection of *Map* DNA in faeces has gained attention recently. Initial difficulties with DNA extraction and purification (Kawaji et al. 2007) have been overcome by using a less labour-intensive DNA purification method which reduces the risk of cross contamination (Plain et al. 2014).

### 2.3.3 Control

Few studies have investigated risk factors for JD in sheep. A study of nine farms documented a relatively higher number of new clinical cases during June-July (i.e. late pregnancy) and December-January (i.e. late lactation/weaning) than during the rest of the year, suggesting that physiological stressors may predispose sheep to disease (Davidson 1970). Merinos are reportedly more susceptible to JD than Romneys (Morris et al. 2006). In Australia relatively high prevalences of JD were observed in the offspring of dams with poor body condition or retarded growth, or when ewes were kept at high stocking rates during lambing (Dhand et al. 2007).

In New Zealand there is no surveillance or systematic national control scheme for JD in sheep. A fact sheet on the management of JD in sheep has been prepared by Beef and Lamb New Zealand and JDRC (Anonymous 2016).

Experience from Australia and elsewhere has shown that test-and-cull or destocking-restocking strategies were ineffective for eradicating JD (Fridriksdottir et al. 2000, Taylor & Webster 2005). Preventive measures other than vaccination were deemed unsuitable on a large scale due to cost and social factors involved (Fridriksdottir et al. 2000, Juste & Perez 2011). In an Australian field trial using a killed vaccine (*Map* 316F strain; Gudair<sup>TM</sup>; Zoetis) in Merino lambs aged 1–3 months from pastoral flocks with 5–15% annual mortality, mortality was reduced by 90% after one year (Reddacliff et al. 2006). Prevalence of *Map* shedding was reduced by 90%, while subclinical infections fell by

66%. Nevertheless, multibacillary lesions were present in all vaccinates dying with JD and they were possibly shedding large numbers of bacteria (Reddacliff et al. 2006). In another Australian study of Merino flocks that had been vaccinating lambs for at least five years with the killed *Map* 316F strain vaccine, the median prevalence of infection, before and after vaccination was 2.72 and 1.99%, respectively (Dhand et al. 2013). While the pre-vaccination infection prevalence was determined using AGID, PFC or both, the post-vaccination prevalence was determined by PFC only. The authors concluded that vaccination must be continued for a number of years for economic return to be achieved. The killed *Map* strain 316F vaccine is licensed for use in sheep in New Zealand. While vaccination-site lesions occur (Bastida & Juste 2011), they have been reported to subside rapidly (Thompson et al. 2002). It was estimated that vaccination was cost-effective if  $\geq 2\%$  of breeding ewes were clinically affected annually (Brett 1998). Evaluation of the cost-effectiveness of control strategies in sheep in New Zealand is currently under way.

## 2.4 Deer

### 2.4.1 Epidemiology

While the New Zealand farmed deer industry commenced in 1972, the first report of JD in deer was in 1986 (Gumbrell 1986). Approximately 96% of *Map* cultures from deer are Type C (de Lisle et al. 2006), which appears to be more virulent than Type S for deer (Mackintosh et al. 2007). Herd-level infection prevalence was estimated at 46–59% (Stringer et al. 2013a, Verdugo et al. 2014a) and 45% of individual one-year-old deer had *Map* cultured from the mesenteric lymph nodes (Stringer et al. (2013a); Table 2.1). Prevalence in both herds and individuals was higher in the South Island than in the North Island. A study of non-randomly selected infected herds, using a deer-specific IgG1 ELISA (Paralisa<sup>TM</sup>; (Griffin et al. 2005)), reported a within-herd seroprevalence of  $>5\%$  in most herds tested, with a few being  $>20\%$  (Griffin et al. 2007).

In contrast with cattle and sheep, in deer the highest incidence of clinical disease was described anecdotally as being in animals from 8-15 months of age, sometimes as outbreaks, but only sporadically in older animals (Mackintosh et al. 2004). In a more definitive study based on an on-farm survey and PFC of 174 herds nationwide, the herd-level incidence

of JD, based on farmer diagnosis conforming to a prescribed case-definition, was up to 21.5% in weaners (0–12 months of age). Median annual incidence of JD in PFC positive herds was 2% in yearling (12–24 month-old) hinds and stags and 1.2% in weaners (Hunnam 2011).

### 2.4.2 Diagnosis

As for cattle and sheep, the lack of sufficiently reliable diagnostic tools to detect latent and subclinical infection constrains control of JD in deer (Table 2.2). A commercial ELISA (Paralisa<sup>TM</sup>), using two antigens, was developed specifically for deer (Griffin et al. 2005). Initial testing indicated that the specificity at a cut point of 50 ELISA units (EU) was 99.5%, and sensitivity using both antigens in series was 91%. However most deer used to estimate sensitivity were clinically affected, which would likely result in a falsely high estimate. Subsequently, the sensitivity and specificity of the ELISA compared with mesenteric lymph node culture in 1–2-year-old hinds was 19 (95% CI = 10–30)% and 94 (95% CI = 93–96)%, respectively, as determined using a Bayesian absence-of-gold standard statistical method Stringer et al. (2013b). Lower specificity compared with ELISA tests in other ruminant species. Faecal culture sensitivity was 77% and specificity 99% for detection of the subclinically infected animals (Stringer et al. 2013b), higher than the sensitivity of the ELISA for that class of animal.

The ELISA has been evaluated to indirectly predict *Map* shedding. Using cut points of >50 EU, >100 EU and >150 EU, the positive predictive values for faecal shedding (threshold  $\geq 104$  *Map/g*), were 0.55, 0.7 and 0.9, respectively. The negative predictive value was 0.9 at all three cut-points (O'Brien et al. 2013). Hence the ELISA can be used with reasonable confidence to predict high shedding when interpreted at high cut points.

A quantitative PCR test can be used to detect presence of *Map* infection at individual animal level. Alone, or interpreted in parallel with ELISA to achieve higher sensitivity, quantitative PCR may be used to detect the highest shedders, which pose the greatest exposure risk to herd mates (O'Brien et al. 2013).

For herd-level screening, Verdugo et al. (2014a) estimated sensitivity and specificity of PFC, using two pools of 10 samples from yearlings, to be 88% and 98%, respectively. Validation of this regime would be required if used for other age groups.

### 2.4.3 Control

As with cattle and sheep, control strategies in deer are aimed at management not eradication, in order to minimise clinical signs on-farm, gross pathology at slaughter and reactors to tuberculosis testing. Control measures will vary depending on the farm situation, and it is recommended that a farm-specific plan is developed (Hunnam & Goodwin-Ray 2010).

An investigation of herd-level risk factors for infection found nine- and four-fold higher infection rates in Canterbury and Southland, respectively, compared with herds in the North Island. Risk was higher on hill and/or high country farms 600 m above sea level compared with farms  $\leq 600$  m, and was lower when there were  $>5\%$ , compared with  $\leq 5\%$ , adult stags in herd, where no clinical cases occurred in the previous year compared with a history of clinical diseases, and where dams were available for stock water compared with no dams (Hunnam 2011).

The importance of culling clinically affected, high shedding deer was shown by a simulation model (Heuer et al. 2012) which suggested that for young deer, culling those with clinical disease within one week reduced overall prevalence to 4%, rather than to 63% if culled after eight weeks. By contrast, culling of adult deer after detection of clinical disease had little impact on overall prevalence and incidence of clinical disease, because relatively few adult deer develop JD.

Vaccination with a killed *Map* strain 316F vaccine (Silirum<sup>TM</sup>) in a randomised clinical trial in young deer in naturally exposed herds, significantly reduced JD incidence by 60%, by approximately 12 months of age (Stringer et al. 2013c), but did not reduce the degree of or proportion of animals shedding. However, quantification of shedding was not undertaken. Stringer et al. (2011) demonstrated that while vaccination interfered with the intradermal tuberculin test for tuberculosis, ancillary testing was able to distinguish cross-reaction due to vaccination. Of greater concern is the observation that vaccine cross-reactivity reduced the sensitivity of the tuberculosis test (Mackintosh et al. 2008).

In 2007, an industry-initiated national abattoir surveillance programme for monitoring of infection with *Map* was established, based on meat inspectors evaluating the presence of mesenteric lymph nodes with a circumference  $>50$  mm (enlarged visceral lymph node), which is approximately 90% predictive of infection with *Map* in deer (Hunnam et al. 2013). The programme is managed by Johnes's Management Limited (JML) and

funded by a levy on each commercially slaughtered deer. Mesenteric lymph nodes from every farmed deer slaughtered commercially are evaluated and those with enlarged visceral lymph nodes, or with typical gross pathology, are recorded as suspect for JD. Other data include farm ID and geo-location, carcass weight, crude age category and sex of the carcass. This information is reported to farmers, with the objective of creating awareness, advising the industry on trends for suspected JD prevalence and ultimately, effectiveness of control measures. Johne's Management Limited also provides educational material for management and maintains and analyses enlarged visceral lymph node data (Norton 2012a).

A consultant network of veterinarians and deer specialists has been established, who have undertaken specific training to address the JD problem on commercial deer farms. They are provided with JD control tools such as epidemiological information, examples of how on-farm risk management plans can be developed, specifics of JD in deer and expert perspectives, to develop individual farm-tailored JD control programmes with farmers who wish to employ this service (Norton 2012b). The strength of this tailor-made control programme is that it can incorporate farms using different farming methods and address factors characterising epidemiology of the disease better. The main control measures that can be undertaken by farmers have been summarised in a technical manual, and include managing risk of *Map* entry onto a farm, reduction of within-herd transmission and optimisation of herd health through feeding and disease prevention (Hunnam & Goodwin-Ray 2010).

Minimising risk of entry of *Map* onto a farm requires farmers to identify possible entry points for infection and minimise those through appropriate management practices. Such entry points may include deer or other livestock purchased from other farms, contact of deer with off-farm livestock including sheep, cattle and goats, or from deer grazed off-farm, and off-farm water sources.

Minimising on-farm transmission of *Map* requires an evaluation of presence and estimation of infection burden on the farm, followed by identification and removal of infected or suspect animals. Transmission should also be minimised by preventing ingestion of organisms from soil, pasture or water contaminated with faeces from infected animals, as well as pre- and postnatal transmission. Management practices may include resting pastures, treatment of contaminated pastures, quarantine of purchased animals on arrival,

**Table 2.1:** Summary of herd or flock prevalence and annual clinical incidence in infected flocks for infection with *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) in different farm animal species in New Zealand.

	<i>Map</i> strain	Observable signs	Reported herd/flock infection prevalence	Annual clinical incidence in infected herds/flocks
Cattle				
Dairy	90% type C <sup>1</sup>	Low BCS in older cows, persistent diarrhoea	Robust data not available	Up to 0.47 (SD 0.765)% <sup>6</sup>
Beef	80% type S <sup>1</sup>	Signs of clinical disease rarely observed	42 (95% CI = 35–50%) <sup>4</sup>	0.21 (95% CI = 0.07–.34)% <sup>7</sup>
Sheep	87% type S <sup>1</sup>	Low BCS of mostly 4-year-old ewes <sup>2</sup> , diarrhoea often absent	76 (95% CI = 70–81%) <sup>4</sup>	0.27 (95% CI = 0.08–0.45)% <sup>7</sup>
Deer	91% type C <sup>1</sup>	Low BCS, all ages, particularly young, diarrhoea of varying intensity and less common <sup>3</sup>	59 (95% CI = 41–78)% <sup>5</sup>	0.42 (95% CI = 0.16–0.65)% <sup>7</sup>
References	<sup>1</sup> Verdugo et al. (2014b)	<sup>2</sup> Davidson (1970), <sup>3</sup> Mackintosh et al. (2004)	<sup>4</sup> Verdugo et al. (2014a), <sup>5</sup> Stringer et al. (2013a)	<sup>6</sup> Hunnam (2014), <sup>7</sup> Verdugo (2013)

BCS = body condition score ; CI = confidence interval ; SD = standard deviation

using only test-negative hinds for breeding and culling offspring from infected hinds.

## 2.5 Discussion

This review focuses primarily on JD control in dairy cattle, sheep and deer in New Zealand. There is no focus on beef cattle and goats, due to the paucity of information, and lack of attention to or apparent insignificance of JD in those industries. In New Zealand livestock farming is predominantly pasture based as the climate favours the growth of green pasture all the year round. This is different to more intensive livestock farming systems practiced in North America. However, several intensive shed based housing can also be now found in New Zealand as the farming systems have further diversified in recent years. Sheep and beef cattle in New Zealand are usually farmed and grazed together as these two species have complimentary nature in terms of pasture and animal health management, a practice that probably increases the likelihood of cross species transmission of *Map* (Verdugo et al. 2014b). Environmental contamination by *Map* may be higher in intensive farming systems compared to extensive pastoral systems (Barkema et al. 2018) and potentially in intensive systems individual animals have a higher exposure to *Map* pathogens.

There are notable differences in how each livestock industry prioritises JD, though control

**Table 2.2:** Post-mortem lesions of infection with *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) in different farm animal species and diagnostic test used in herds and individual animals in New Zealand.

	Post-mortem lesions			Diagnostic tests	
	Small intestines	in-lymph nodes	Lymph nodes	Herd-flock level	Individual animal
Cattle	Thickened and corrugated	Extensive necrosis of mesenteric lymph nodes often absent		PCR assay and serum/milk ELISA <sup>2</sup>	clinical cases – serum ELISA, faecal culture <sup>5</sup> ; subclinical cases– faecal culture
Sheep	Thickening not always present	Extensive necrosis of mesenteric lymph nodes often absent		PFC, ELISA <sup>3</sup>	clinical cases– serum ELISA, necropsy, histopathology <sup>6</sup> , PCR <sup>7</sup> ; subclinical cases– faecal culture histopathology
Deer	Thickening not always visible	Extensive necrosis in mesenteric lymph nodes. Lymphatic cording is common <sup>1</sup>		PFC <sup>3</sup> , ELISA for screening at cut point >100 EU <sup>4</sup>	clinical cases– serum ELISA; subclinical cases–faecal culture, ELISA <sup>8</sup>
References		<sup>1</sup> de Lisle et al. (2003)		<sup>2</sup> Anonymous (2015a), <sup>3</sup> Verdugo et al. (2014a), <sup>4</sup> O'Brien et al. (2013)	<sup>5</sup> Norton et al. (2010), <sup>6</sup> Anderson & Heuer (2016), <sup>7</sup> Plain et al. (2014), <sup>8</sup> Stringer et al. (2013b)

EU = ELISA units; PFC = pooled faecal culture

**Table 2.3:** Strategies used for control of Johne's disease in different farm animal species in New Zealand

	Control strategy	Vaccines	Surveillance
Cattle			
Dairy	Voluntary participation of farmers using risk management tools <sup>1</sup>	Restricted due to interference with Tb testing	No national surveillance
Beef	No control due to low incidence	Restricted due to interference with Tb testing	No national surveillance
Sheep	Voluntary, long term use of vaccination recommended based on >2% annual loss of breeding ewes due to clinical disease <sup>2</sup>	No restriction: vaccine available	No national surveillance
Deer	Voluntary participation of farmers in programmes coordinated by JML	Vaccine effective in prevention of clinical disease in young deer <sup>3</sup> . Vaccine can possibly interfere with Tb control <sup>4</sup>	Abattoir-based national surveillance established in 2007 <sup>5</sup>
References	<sup>1</sup> Anonymous (2015b), <sup>2</sup> Brett (1998)	<sup>3</sup> Stringer et al. (2013b), <sup>4</sup> Mackintosh et al. (2008)	<sup>5</sup> Norton (2012a)

JML = Johne's Management Limited; Tb = bovine tuberculosis

is voluntary in all (Table 2.3). The management strategies developed for dairy cattle were largely based on data from overseas intensive, rather than pastoral systems, along with first principles of disease control, because there are limited New Zealand-specific data. The programme is not comparable with those implemented in some other developed countries (Geraghty et al. 2014). It is neither systematically promoted nor financially or institutionally supported, and, to date lacks evaluation of its effectiveness in a New Zealand pastoral context.

In principle, control should either minimise introduction of *Map* to non-infected herds or, if the herd is already infected, minimise exposure of susceptible animals (Nielsen 2014). Two key areas for JD control in the dairy industry in New Zealand are reduction in farm-level prevalence, and milk treatment to ensure dairy products are *Map*-free (Burton & Voges 2002). The former area requires knowledge about management factors specific to New Zealand pastoral dairy systems that are associated with high prevalence. The two available studies of New Zealand dairy herds (Norton et al. 2009, Hunnam 2014) provide basic information that needs to be confirmed by robust, longitudinally collected data to establish cause-effect relationships.

PTb control in sheep is less studied in New Zealand than in dairy cattle and deer. While the majority of flocks are infected but have low JD incidence, some farms, particularly fine wool producers, are disproportionately affected leading to high ewe mortality (Anderson & Heuer 2016). In such cases vaccination of replacement lambs appears to be the most suitable tool for delaying the onset, and reducing the number of JD cases. However, longitudinal studies of vaccine efficacy and benefit-cost analyses should be given priority. Control in deer is more proactive and the deer industry is better supported by research-led programmes and surveillance than other livestock industries. Nevertheless, published research is required to validate the slaughterhouse surveillance system as a proxy for farm-level infection-status. The potential for vaccination has been shown, and its use may be permitted in future when tuberculosis control is achieved.

## 2.6 Conclusions

In general, JD is common and widespread geographically in New Zealand. There is scarcity of robust data on management factors associated with higher prevalence of in-

fection and JD in New Zealand in all livestock industries. Little research attention has been given to JD in the beef and dairy goat industries. This limits understanding of the economic justification for control measures on-farm, and their effectiveness nationally in all species. Hence, while current control initiatives are based on first principles, supported in part by overseas data, many aspects of control of JD in this country are somewhat subjective. Control is largely aimed at maintaining livestock productivity and welfare by limiting clinical disease incidence. There is variation between industries in the means for control and the extent to which they have been adopted or have addressed the issue. The dairy industry has adopted a voluntary programme encouraging its participants to use management strategies to assist control at the individual farm level but no mechanism exists for its systematic application, adoption or evaluation. While vaccination has been shown to be at least partially effective in many species, its adoption in cattle and deer is limited by potential conflict with tuberculosis control objectives. Despite vaccination use in the sheep industry, cost-effectiveness needs to be evaluated to inform decisions about its adoption at farm level. Arguably, the deer industry has been the most proactive in addressing JD, and is the only industry to attempt national surveillance. Both biological and economic effectiveness of activities and recommendations in all industries discussed here now need to be evaluated, to inform future initiatives for JD control.

## **2.7 Acknowledgements**

The authors thank Kaylene Larking, JDRC Manager, and Dr. Rory O'Brien of the Disease Research Laboratory, University of Otago, for comments on the manuscript. This review was funded by the JDRC.



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## Economic cost of ovine Johne's disease in clinically affected New Zealand flocks and benefit-cost of vaccination

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**Abstract** – The aims of this study were to estimate the on-farm economic cost of ovine Johne's disease (OJD) based on collected incidence and mortality data, and the benefit-cost of OJD vaccination in typical OJD affected flocks in New Zealand after having vaccinated for a number of years. Owners of 20 sheep breeding and finishing farms known to be clinically affected by ovine Johne's disease in New Zealand participated in the study and were monitored for up to two years. Farms were categorized as fine-wool (Merino, Half-Bred, Corriedale, n = 15), and other breeds (Romney, composite breeds, n = 5). Ovine JD was confirmed by gross- and histo-pathology in 358 ewes culled due to chronic progressive wasting. An additional 228 ewes with low body condition score (BCS), but not targeted for culling, were tested with ELISA to estimate the proportion of OJD in ewes in the lower 5% BCS of the flock. Calculations were done separately for fine-wool and other breeds. Based on the data, mortality due to OJD, its associated cost and the benefit-cost of vaccination were evaluated for a hypothetical farm with 2,000 breeding ewes by stochastic simulation. Total ewe mortality was similar in fine-wool and other breeds, but the estimated mortality due to OJD was 2.7 times as high in fine-wool (median 1.8%, interquartile range (IQR) 1.2–2.7%) than other breeds (median 0.69%, IQR 0.3–1.2%), but with large variation between farms. ELISA results demonstrated fine-wool sheep had a higher seroprevalence than other breeds (39%, 95% CI 18–61% vs. 9%, 95% CI 0–22%). Stochastic modelling indicated that the average annual cost of mortality due to OJD in a flock of 2,000 ewes was NZ \$13,100 (IQR \$8,900–18,600) in fine-wool and NZ \$4,300 (IQR \$2,200–7,600) in other breeds. Vaccinating replacement lambs against OJD may be cost-effective in most flocks when the pre-vaccination annual ewe mortality due to OJD is >1%. To make the best-informed decision about vaccination it is therefore essential for farmers to accurately diagnose OJD to establish incidence.

**Key words:** ovine Johne's disease, economics, mortality, vaccination, New Zealand

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### 3.1 Introduction

Sheep farming contributes significantly to the New Zealand economy. The sheep industry earned NZD 3.7 billion in the year ending in June 2016, primarily through exporting meat (lamb and mutton) and raw wool fibre (Beef and Lamb New Zealand 2017).

Conventionally New Zealand sheep production is entirely pasture-based and characterised by large flocks. Meat breeds such as Romney and composite (mixed) breed sheep comprise more than 50% of the national population while fine-wool breeds such as Merino and Corriedale comprise less than 10% (Beef and Lamb New Zealand 2017). Generally, the health status of sheep in New Zealand is good although several infectious diseases, including paratuberculosis, are endemic in the country (Orr 1998).

Paratuberculosis, often synonymously referred to as Johne's disease (JD), is a chronic bacterial disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) in ruminants. In this study the term ovine Johne's disease (OJD) refers to clinical disease, usually resulting in mortality. *Map* infection is widespread in New Zealand with at least 75% of sheep flocks infected (Verdugo et al. 2014a). About 2–6% of flocks were believed to be clinically affected (Bryan & Cresswell 2012) and annual incidence of clinical disease within affected flocks was estimated to be about 1% or lower (West 1997, Verdugo 2013). However, those rates were not supported by longitudinal flock monitoring data, hence no robust estimates of incidence are available. Nevertheless, the incidence and prevalence of *Map* infection and disease is likely to be economically important on some farms. Affected farms suffer economic losses due to sheep deaths and reduced production (Morris et al. 2006).

There are limited data on economic cost of OJD to the New Zealand sheep industry. The only analysis, which was based on a simulation rather than physically monitored production effects, was published almost two decades ago (Brett 1998). It assumed that if 6% of flocks were infected the estimated minimum annual cost to the industry was NZ \$0.9 million. Alternatively, if 70% of flocks were infected, a scenario that is more representative of the current prevalence (Verdugo et al. 2014a), the estimated annual loss would have been NZ \$9.9 million (Brett 1998).

While some, albeit limited, data now exist for the production effects of clinical JD on

some New Zealand sheep farms (Morris et al. 2006), there is no well-researched current assessment of the economic cost of OJD or *Map* infection at farm level. A vaccine is registered in New Zealand to control OJD. However, the benefit-cost of OJD vaccination is unknown. Hence this study aimed to investigate mortality due to OJD and to evaluate the cost of OJD in a typical infected flock of fine-wool or other breeds under pastoral conditions in New Zealand using longitudinal data. Based on the results, an additional aim was to estimate the cost-benefit of vaccination.

## 3.2 Materials and methods

All manipulations performed on animals were approved by the Massey University Animal Ethics Committee (MUAEC 12/75). Where expert opinion was required, they were those of co-authors P. Anderson and A. Ridler.

### 3.2.1 Farms and data collection

Twenty sheep farms were enrolled in the study comprising fifteen fine-wool (Merino, Half-Bred, and/or Corriedale) and five other (Romney and/or composite) breeds. All fine-wool farms were in the South Island while the other breed farms were in the North Island of New Zealand. A half-bred, classified as a fine-wool breed in this study, is a cross-breed between a Merino ewe and a Romney or English Leicester ram. A composite breed, classified as other breed, is a combination of diverse mutton breeds.

Farms were enrolled between August 2012 and July 2013 and monitored until to June 2014 to obtain tallies (counts) of ewes at mating, scanning, set-stocking for lambing, and tailing. They also contributed ewes for necropsy and/or ELISA. Complete tallies were provided from 17 farms (13 fine-wool and 4 other), which had flock sizes range from 785 to 20,104 ewes. Of these 17 farms, 13 were monitored over two years and four over one year, providing ewe tally data from more than 100,000 ewes over a total of 30 farm-years (Table 3.1). In addition to the ewe tally data, these farms also provided annual tailing data, which represented the number of lambs tailed (at 3–6 weeks of age) per ewe per farm-year. This tailing percentage was used as a proxy for lambing percentage, a parameter

used in the economic analyses described later. The remaining three farms did not provide tallies and only contributed ewes for necropsy or ELISA.

### 3.2.2 Annual mortality due to OJD

We estimated the annual ewe mortality rate due to OJD separately for each breed-type based on three sequential steps. The first was the overall incidence rate of annual ewe mortality determined from ewe tallies (ewes that died during the year/ewes present at start of the season) at mating in March-May in the year of enrolment, at ultrasound scanning for pregnancy in June-July, at set stocking for lambing in August-September, at tailing/weaning in October-January, and at the next mating in March. Ewes missing or unaccounted for at tallies were assumed to have died. The overall ewe mortality per farm-year was the cumulative number of missing ewes divided by total number of ewes at the first count.

The second step established the proportion of total mortality that was 'likely OJD-related'. It was required to correct for the potential ewe-selection bias for necropsy (step 3). The estimate was based on records from the 14 farms from which farmer-diagnosed causes of death were available. Causes were categorised as either 'likely OJD-related' or 'likely OJD-unrelated'. The 'likely OJD-related' category was regarded as a reasonable representation of the ewes submitted to necropsy by farmers and comprised three types of mortality causes: 'possibly Johne's', 'dog tucker' (used as dog food) and 'found dead on the farm'. 'Likely OJD-unrelated' causes of deaths recorded by farmers, on the other hand, included a wide range of causes including, but not limited to accidental deaths, poisoning, drowning, fly strikes, injuries, metabolic diseases, sudden deaths, mastitis, hernia, taruma and dystocia.

The third step was the proportion of 'likely OJD-related' ewes (step 2) confirmed to be OJD by post-mortem. It was based on necropsy of 358 ewes from 19 farms (15 fine wool and four other breed ) with a body condition score (BCS) of one on a scale of 1–5. These ewes were selected by farmers and necropsied by local veterinarians. Gross pathological findings were confirmed by histology of fixed ileocecal valve and lymph node, distal ileum, terminal jejunum and mesenteric lymph node, and classified as either OJD positive, i.e., conforming to lesion categories one to three described by Prez et al.

(1996), or negative.

The overall annual ewe mortality rate due to OJD was calculated for each breed type as = overall incidence rate of annual ewe mortality rate (step 1) x proportion of 'likely OJD-related mortality' (step 2) x proportion of confirmed-OJD mortality (step 3).

### 3.2.3 Proportion of OJD-affected ewes

An OJD-affected ewe was defined as one which was presumed would progress to clinical OJD, being of low body condition as identified by farmers at the time of and tested positive by serum ELISA. To estimate the proportion of affected ewes, a sample of 228 ewes [range 2–69 per flock from 15 farms (11 fine wool and 4 other breeds)] with BCS  $\leq 1.5$  were tested by serum ELISA at New Zealand Veterinary Pathology Limited. Ewes tested with ELISA were not targeted for culling *per se*, but represented ewes with low body condition in the flock. Selection of ewes sampled for ELISA testing was done jointly by farmers and their vets when the latter visited the farm for sampling ewes for post-mortem. Based on expert opinion, we assumed that 5% of ewes of a typical flock in either breed category would have BCS  $\leq 1.5$ . If an ELISA positive ewe was necropsied it was included in the necropsy group. An estimate of the number of OJD-affected ewes in a flock was calculated from the proportion of ewes with BCS  $\leq 1.5$  that were ELISA positive among the 5% of ewes with low BCS.

Data from OJD-affected ewes were used to estimate pre-clinical loss (poor reproductive performance, lower carcass weight of cull-ewes). The pre-clinical loss was calculated by the difference in productive lifetime among the necropsied ewes (i.e., age at culling/death, which was estimated based on ear marks or ear tags) between OJD-confirmed and non-OJD ewes, multiplied by an assumed average annual profit per ewe of NZD 40 for fine-wool and NZD 35 for other breeds based on expert opinion, the assumed proportion of live ewes in a flock with BCS  $\leq 1.5$  (i.e., 5% of the total flock for both breed categories based on expert opinion) and the proportion of ELISA positive ewes.

### 3.2.4 Data processing and statistical analyses

Data on ewe mortality, necropsy, histopathology and serology were stored in an online database called IRIS maintained by the Epicentre at Massey University, New Zealand.

All calculations were done separately for fine-wool and other breeds. Statistical analyses were conducted using an open source computer program R, version 3.1.3 (2015-03-09) for Windows (R Development Core Team 2015). A probability of  $p < 0.05$  was considered statistically significant and confidence intervals were computed at 95%.

### 3.2.5 Economic analysis

To estimate economic effects and benefit-cost analysis of vaccination, various cost and revenue parameters were calculated. If necessary data were not available from this study or literature, they were based on expert opinion. These parameters and assumptions of stochastic modelling of OJD economics are presented in Table 3.2. The vaccine efficacy estimate used in this study was from a clinical trial on self-replacing Merino farms that had  $>5\%$  annual OJD-related mortalities in Australia (Reddacliff et al. 2006). We assumed that only female replacement lambs were vaccinated. In the calculation, 50% of other breed lambs were vaccinated while 60% of fine-wool were vaccinated because of lower reproductive rates in these breeds. Likely variable cost and revenue parameters were subjected to stochastic uncertainty (Table 3.2). Parameters from expert opinion that were held constant during stochastic modelling are shown in Table 3.3. Economic effects were estimated for a hypothetical flock of 2,000 breeding ewes, a number considered representative of an average commercial sheep flock in New Zealand.

In addition to OJD-specific mortality, economic outcomes linked to OJD mortality, overall OJD-related loss at farm-level and benefit-cost of vaccination were based on stochastic simulation involving 10,000 random draws. The use of stochastic simulation accounted for uncertainties around parameters used for estimation of OJD-specific mortalities, economic outcomes linked to OJD mortality, as well as production cost and revenue figures taken from other sources mentioned above. Consequently, outputs are shown as distributions after having adjusted for uncertainties around the input parameters used for deriving them. Economic model and R codes used to estimate OJD mortality and the economic outcomes of vaccination is presented in Appendix A.

## 3.3 Results

Thirteen fine-wool and four other breed properties provided ewe tallies, scanning and tailing data and submitted ewes for necropsy. Of the farms that did not provide tally records, the two fine-wool farms contributed sheep for necropsy, while the one other breed farm provided serum samples for ELISA but neither tallies nor ewes for necropsy.

Average tailing percentage per ewe was 103% (range between farms 66–135%), but these estimates were highly dependent on data from fine-wool farms as only two farm-year data sets for tailing percentage were available for the other breed.

### 3.3.1 Ewe mortality

The crude incidence rate of annual ewe mortality (step 1) was 7.6% (n = 109,320 ewes, 8,287 deaths; CI 6.5–8.6%). No significant difference was observed between fine-wool (7.6%, CI 6.3–8.8%) and other breeds (7.6%, CI 5.2–9.9%).

In step 2, 36% (SD 14%) of all ewe deaths were likely OJD related in fine-wool breeds compared with 21% (SD 25%) in other breeds.

Among necropsied ewes (step 3), OJD was the cause of wasting or death in 68% (CI 60–79%) of fine-wool vs. 27% (CI 4–50%) of other breeds ( $p < 0.001$ ). In total, OJD was considered to be the major cause of death in 218 out of 358 necropsied ewes, which had more widespread lesions in multiple areas of the intestine including the Peyer's patches and associated mucosa and conformed to type 2 (n = 3 ewes) and 3a-c (n = 215 ewes) lesions described by Prez et al. (1996). The age distribution of necropsied ewes was normal, but ewes with confirmed OJD were 0.48 years younger ( $p = 0.001$ ) than those dying of other causes, after controlling for the effect of farm. The lower age of confirmed cases was independent of breed since the interaction term between breed and OJD outcome was not significant.

### 3.3.2 ELISA

Of the 228 ewes tested with serum ELISA, 22.3% were positive overall, with 40% (CI 18–61%; n = 101) of fine-wool and 9% (CI 0–22%; n = 127) of other breed ewes testing

positive ( $p < 0.001$ ).

### 3.3.3 Stochastic simulation

Based on the stochastic simulation, annual OJD mortality was 2.7 times as high in fine-wool (median 1.83%; IQR 1.2–2.7%) than in other breeds (median 0.68%; IQR 0.3–1.2%) (Figure 3.1).

Stochastic analysis and assumptions for estimation of benefit-cost of vaccination is presented in Table 3.4. Annual economic cost due to OJD mortality and benefit-cost ratio of vaccination were highly correlated, with positive financial return occurring from vaccination when the annual mortality rate due to OJD was more than 1% (Figure 3.2). The resulting distribution of the benefit-cost of OJD vaccination is illustrated in Figure 3.3.

For farms with fine-wool breeds, the average incidence rate of 1.8% OJD mortality per year resulted in an average return of NZ \$4.2 (IQR \$2.8–6.1) for NZ \$1 invested in vaccination. The equivalent return for other breeds, with an average of 0.68% OJD mortality, was NZ \$1.6 (IQR \$0.8–2.9). Returns above the breakeven point were realised in at least 95% and 65% of the simulated fine-wool and other breed flocks, respectively. Annual ewe mortality due to OJD was the important determinant of economic return from vaccination as 10% increase in the average OJD attributable mortality would increase the benefit cost ratio of vaccination to 7.3 and 3.3 in fine-wool and other breeds respectively.

Costs due to OJD-associated lamb, ewe, replacement and pre-clinical loss in the two breed categories are shown in Table 3.5. For farms with the average incidence rate of OJD mortality per year the annual loss for a farm with 2,000 fine-wool ewes would be NZ \$13,100 (IQR \$8,900–18,600) and NZ \$4,300 (IQR \$2,200–7,600) for other breeds. Differences in annual economic loss between the two breed categories were attributable to breed-specific differences in reproductive performance and value of animal for sale.

**Table 3.1:** Flock tallies of study farms by breed.

Farm	Breed	Years monitored	Ewes mated	Total missing	Ewe mortality(%)
Fine-wool breed					
A	Corriedale	2012-2013	1040	29	2.8
		2013-2014	1091	78	7.0
B	Half-bred	2012-2013	2,130	136	6.4
		2013-2014	2,316	154	6.6
C	Merino	2012-2013	3,106	209	6.7
		2013-2014	3,320	260	7.8
D	Merino	2012-2013	5,300	512	9.7
		2013-2014	4,631	552	11.9
E	Merino	2012-2013	3,670	135	3.7
		2013-2014	3,867	151	3.9
F	Merino	2012-2013	3,459	212	6.1
		2013-2014	3,595	177	4.9
G	Merino	2012-2013	2,763	195	7.1
		2013-2014	2,860	203	7.1
H	Merino	2012-2013	8,402	1317	15.7
		2013-2014	7,909	461	5.8
I	Merino	2013-2014	3,926	254	6.5
J	Merino	2013-2014	4,699	338	7.2
K	Merino	2012-2013	1,780	89	5.0
		2013-2014	1,807	77	4.3
L	Merino	2012-2013	2,590	120	4.6
		2013-2014	2,668	132	4.9
M	Merino	2013-2014	2,244	212	9.4
Other breed					
N	Romney	2012-2013	2,010	226	11.2
		2013-2014	2,190	73	3.3
O	Romney	2013-2014	20,104	1498	7.5
P	Composite	2012-2013	2,064	167	8.1
		2013-2014	2,104	172	8.2
Q	Composite	2012-2013	890	93	10.4
		2013-2014	785	55	7.0

**Table 3.2:** Calculated and expert-opinion-based parameters and assumptions used for stochastic simulation modelling of OJD economics.

Measurement	Distribution	Parameters of distribution		Assumptions about parameters		Source
		Fine-wool	Other breeds	Fine-wool	Other breeds	
Annual overall mortality	beta	a = 8.717 b = 94.8221	a = 10.2378 b = 113.3121	mode = 0.076 95% sure <0.133	mode = 0.076 95% sure <0.127	calculated
OJD as suspected by farmers	beta	a = 3.9543 b = 6.2522	a = 1.4972 b = 2.8591	mode = 0.36 95% sure <0.64	mode = 0.211 95% sure <0.77	calculated
OJD confirmed by necropsy	beta	a = 4.9044 b = 2.711	a = 3.3551 b = 7.1501	mode = 0.6813 95% sure >0.3539	mode = 0.2698 95% sure <0.565	calculated
Average number of lambs docked per ewe per farm year (lambing percentage)	normal	mean = 1 sd = 0.17	mean = 1.31 sd = 0.05	Na	Na	calculated
Proportion of lambs with delayed finishing	normal	mean = 0.5 sd = 0.05	mean = 0.5 sd = 0.05	Na	Na	expert opinion
Days to finish post-weaning	normal	mean = 240 sd = 24	mean = 100 sd = 10	Na	Na	expert opinion
Death rate weaning to finishing (i.e., when lamb has reached marketable weight of 40–50 kg)	beta	a = 6.4243 b = 176.3867	a = 6.4817 b = 214.7868	mode = 0.03 95% sure <0.06	mode = 0.025 95% sure <0.05	expert opinion
Annual ewe replacement rate	beta	a = 3.8761 b = 9.6284	a = 3.8761 b = 9.6284	mode = 0.25 95% sure <0.5	mode = 0.25 95% sure <0.5	expert opinion
Proportion of live ewes in low BCS ( $\leq 1.5$ ) that tested ELISA positive	beta	a = 3.0818 b = 3.1668	a = 15.41 b = 130.70	mode = 0.39 90% sure <0.64	mode = 0.09 95% sure <0.26	calculated
Proportion of ewes with low BCS ( $\leq 1.5$ )	beta	a = 6.1946 b = 99.6983	a = 6.1946 b = 99.6983	mode = 0.05 95% sure <0.1	mode = 0.05 95% sure <0.1	expert opinion
Profit per ewe per year	normal	mean = 45 sd = 4.5	mean = 35 sd = 3.5	Na	Na	expert opinion
Productive years lost due to OJD	normal	mean = 0.48 sd = 0.048	mean = 0.48 sd = 0.048	Na	Na	calculated
Vaccine efficacy in terms of reducing mortality	beta	a = 5.3842 b = 1.4871	a = 5.3842 b = 1.4871	mode = 0.9 95% sure >0.5	mode = 0.9 95% sure >0.5	(Dhand et al. 2010)

Na = not applicable; BCS = body condition score; Beta parameters (a and b) input variables were estimated using the free software BetaBuster

**Table 3.3:** Revenue and production cost parameters based on expert opinion. These parameters were held constant for simulation modelling of OJD economics.

Assumptions	Fine-wool breeds	Other breeds
Revenue from meat per lamb sold (NZ \$)	90	90
Revenue from fleece per ewe (NZ \$)	40	17
Revenue from fleece per lamb (NZ \$)	40	0
Revenue from meat/salvage ewe (NZ \$)	50	70
Revenue from fleece/salvage ewe (NZ \$)	40	17
Cost of a replacement ewe (NZ \$)	105	105
Health and feed cost per day to finish (NZ \$)	0.11	0.11
Proportion of ewes with BCS <1.5	5%	5%
Cost of vaccine per dose (NZ \$)	3.5	3.5
Labour cost of vaccination per ewe (NZ \$)	0.5	0.5
Proportion of ewe lambs born that were vaccinated	60%	50%

**Table 3.4:** Stochastic analysis, and assumptions used, for estimation of benefit-cost of vaccination of lambs in a hypothetical fine-wool and other breed flock of 2,000 adult ewes, based on calculated mortality rates from this study and 90% vaccine efficacy for reducing mortality.

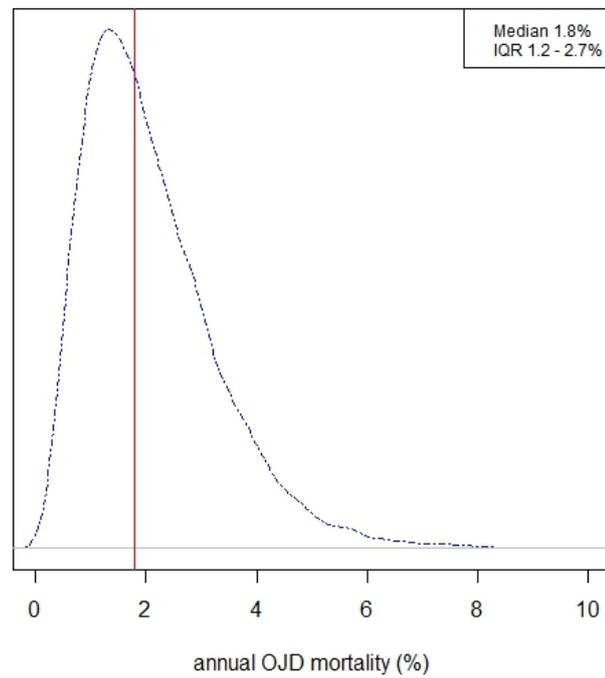
Measurements	Fine-wool breeds	Other breeds <sup>1</sup>
Cost of vaccination		
% ewe lambs vaccinated	60%	50%
No. ewe lambs vaccinated	600	500
Total lamb vaccine cost (NZ \$)	2,400	2,000
Benefit from vaccination		
Achievable benefit (in thousands NZ \$)	0.1 (6.7–14.8)	3.3 (1.6–5.9) <sup>2</sup>
Benefit-cost ratio (BCR)	4.2 (2.8–6.1)	1.6 (0.83–2.9) <sup>2</sup>

<sup>1</sup> due to few farms contributing data, estimates for 'other breeds' are farm specific, and not necessarily population average. <sup>2</sup> figures represent median and range within parenthesis represent the interquartile range.

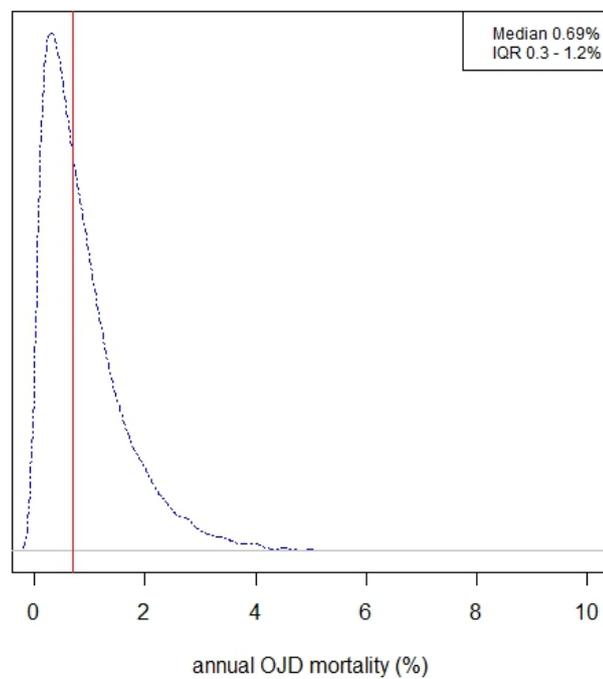
**Table 3.5:** Median (and interquartile range) annual cost of OJD for a hypothetical farm of 2,000 fine-wool or other breed ewes at average annual mortality rates observed in this study.

Measurements	Fine-wool breeds	Other breeds <sup>1</sup>
Annual OJD mortality (%)	1.83 (1.2–2.7)	0.68 (0.33–1.2)
Ewe loss (in thousands NZ \$)	3.3 (2.1–4.9)	1.2 (0.6–2.1)
Lamb loss in terms of opportunity loss due to ewes with OJD (in thousands NZ \$)	4.2 (2.7–6.3)	1.5 (0.7–2.7)
Ewe replacement cost (in thousands NZ \$)	3.8 (2.5–5.7)	1.4 (0.7–2.5)
Cost of preclinical OJD (in thousands NZ \$)	1.3 (0.9–2.0)	0.12 (0.08–0.18)
Total loss due to OJD (in thousands NZ \$)	13.1 (8.9–18.6)	4.3 (2.2–7.6)
OJD production cost per ewe (NZ \$)	6.5 (4.4–9.3)	2.1 (1.1–3.8)

IQR = inter quartile range

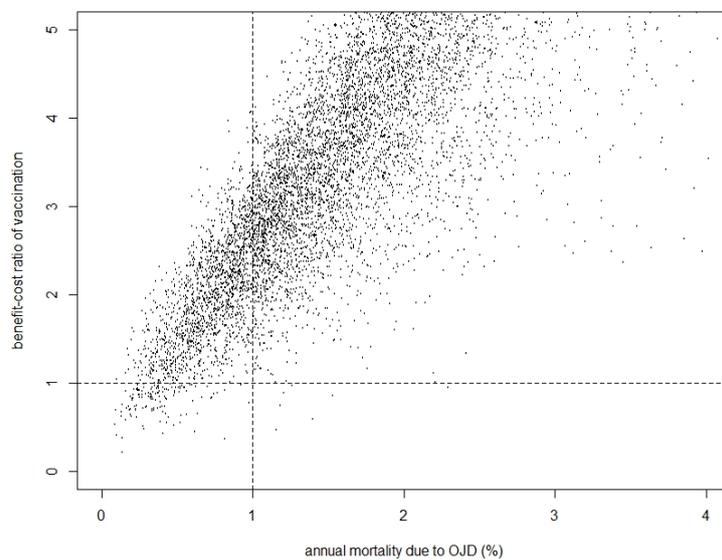


(a)

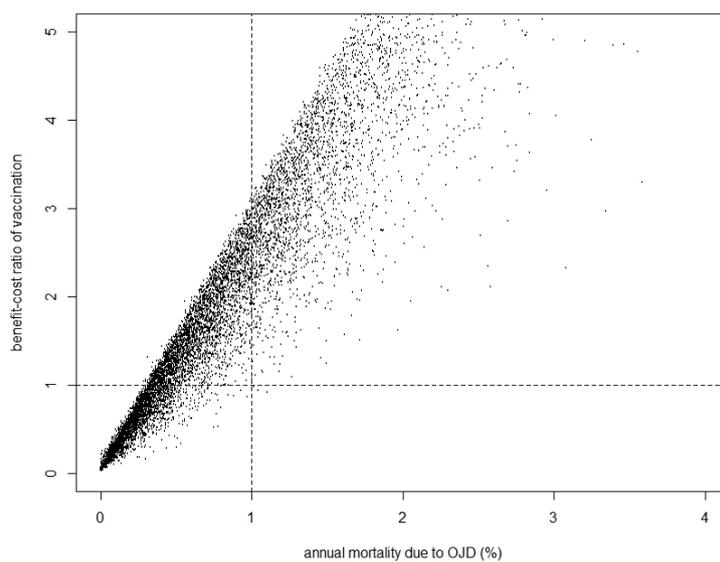


(b)

**Figure 3.1:** Density plots of estimated annual OJD mortality of ewes in fine-wool (a) and other breed (b) flocks and interquartile range (IQR, 25th-75th percentiles). The vertical line represents the median.

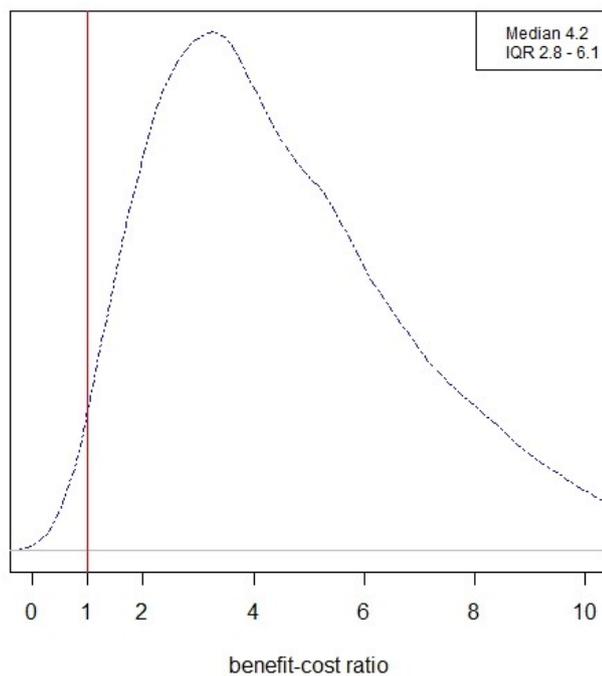


(a)

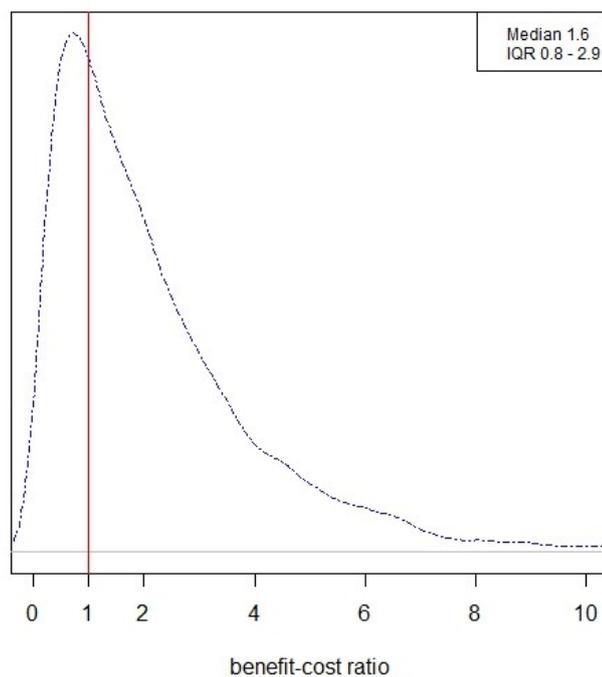


(b)

**Figure 3.2:** Scatter plots of correlation between annual mortality due to OJD and benefit-cost ratio of vaccination in fine-wool (a) and other breed (b) flocks. The dotted horizontal line represents the breakeven point above which vaccination is beneficial and the dotted vertical line represents the cut point of annual mortality above which most of the flocks have a benefit-cost ratio above one.



(a)



(b)

**Figure 3.3:** Density plots of benefit-cost ratio in fine-wool (a) and other breed (b) flocks interquartile range (IQR, 25th-75th percentiles). The vertical line represents the breakeven point where benefit-cost ratio is one.

### 3.4 Discussion

In this study mortality rate in fine-wool ewes was not different from that in other breeds, but the rate attributable to OJD was more than twice as high in fine-wool breeds, which suggested higher susceptibility of the latter category ewes to clinical OJD. This was not unexpected as the majority of fine-wool farms in this study were Merino farms and earlier studies from New Zealand and overseas have reported Merino breeds being more susceptible to mortality due to OJD than other breeds (Morris et al. 2006, Lugton 2004). The stochastic analysis showed it would be more economical to vaccinate replacement lambs if the pre-vaccination annual ewe mortality is  $>1\%$ .

Large between-farm variation of OJD-specific mortality was observed for fine-wool breeds, though sample size for other breeds was limited and there was considerable uncertainty about estimated total mortality. Variable OJD mortality indicates that intervention is not warranted on every fine-wool farm. Hence, farm-specific OJD incidence should be objectively and reliably established before interventions such as vaccination are implemented, particularly when economic return is an imperative. The relatively small variation in OJD mortality for other breeds was possibly a chance effect due to few farm-years data, hence more information about other breeds is required before strong inferences can be drawn.

Ewe death rate due to OJD was estimated indirectly based on three mortality categories. Ewes that farmers presented as terminal culls for necropsy after a chronic condition were deemed 'possibly OJD-related culls'. Other categories were ewes in extremely poor condition killed for 'dog tucker', and those found dead on pasture were also considered possible OJD cases. It was assumed that the latter categories at least partially represented ewes that a farmer would otherwise have presented for necropsy. While this was subjective and therefore not always correct, it was considered to be a reasonable interpretation within the logistic limits of the study. The proportion of 'possibly OJD-related culls' was considered a reasonable means of establishing that ewes subjected to necropsy represented all ewes that died or were culled due to terminal conditions.

Ewes classified as OJD-affected were those with low BCS, but not marked for culling. They comprised a small proportion of any flock. For economic analyses it was deemed appropriate to consider that they were expected to have a shorter life-span than ewes with

higher BCS. This group of ewes therefore contributed to the overall OJD-specific loss tallies, in conjunction with culled/dead ewes.

Sensitivity of the serum ELISA test is higher in animals showing symptoms of disease than in latently infected animals (Sergeant et al. 2003). While specificity of this assay is high at approximately 99% (Hope et al. 2000), thereby limiting false positive misclassification of ewes clinically affected by OJD, its sensitivity may range from 22–46% in latently infected clinically healthy sheep (Hope et al. 2000). This low probability of unaffected ewes testing positive by ELISA was helpful for the economic analysis in that most infected animals that were not yet pre-clinical OJD cases would be excluded. This supports that the ELISA was a suitable test for estimating the proportion of OJD-affected live ewes.

To estimate the cost-effectiveness of vaccination, we used data on vaccine efficacy reported in the literature (Reddacliff et al. 2006). Gudair<sup>TM</sup> (*Map* 316F strain, Zoetis, Australia), which is registered for use in sheep in New Zealand, was evaluated by clinical trial on three Merino farms in Australia by Reddacliff et al. (2006) who reported 90% reduction in OJD mortality after one year compared with controls. In a clinical trial of young deer in New Zealand, vaccine efficacy of 60% was reported in terms of decreasing incidence of clinical disease (Stringer et al. 2013c). To account for the possible variation in vaccine efficacy between farms, vaccine efficacy along with other variable parameters were subjected to stochastic simulation.

In this simulation, we estimate the benefit-cost analysis of vaccination at a time when OJD had reached a state of equilibrium (constant prevalence of infection). It is therefore a snap-shot and ignores infection dynamics over time, and thus the time during which replacement lambs were vaccinated, but no benefits were received due to ongoing mortality of adult ewes. Hence, based on this study we can only get an estimate of the economic return after the full-effect of vaccination has been realised. Accounting for the real and discounted return over time would reduce the benefit-cost because a farmer would have to invest in vaccinating the flock for number of years before OJD was sufficiently controlled to prevent most or all of the economic loss attributable to the disease.

Literature review suggests that as annual mortality due to OJD increases, the time required for obtaining a positive return on vaccination investment decreases. Nevertheless, direct comparison between studies is complicated due to differences in model types, definition

and number of model parameters as well as sheep production systems in the country of study.

A recent study modelling OJD in New Zealand Romney flocks (Marquetoux 2017), reported mixed-aged ewe flocks with 1% OJD mortality incidence per year might reach the breakeven (when the annual net profit of vaccination becomes positive), after five years. However, depending on the annual incidence of OJD, it might take several more years for the cumulative benefit of vaccination to be higher than the cumulative cost of vaccination, for example 30, 15 and 10 years for 0.97%, 1.1% OJD and 1.27% OJD incidence, respectively.

Bush et al. (2008) modelled the benefit of OJD vaccination over 20 years in Australian Merino flocks with different initial levels of annual OJD mortality. They suggested in most cases the breakeven point may be reached in four years if the initial disease mortality was high ( $>7\%$ ), 5 years if the mortality was medium (3–7%), or 10 years if the mortality was lower ( $<3\%$ ).

Although post-vaccination lesions were reported to persist for up to four years, they did not result in carcass losses or downgrading (Reddacliff 2005). Hence, no cost was attributed to trimming or downgrading of carcasses during processing in our stochastic model. Regulations governing the management of OJD vaccinated stock in New Zealand have recently been changed such that trimming or detaining of carcasses is no longer prescribed.

The large between-farm variation for most input parameters including mortality and replacement rates and estimated vaccine cost-effectiveness generated by our simulation will not hold for every farm. Hence many lamb-producing farms in particular (i.e. referred to as 'other breeds' in this study) would likely not profit from vaccination. Our economic analysis indicated that vaccination may be advisable and cost effective in farms where OJD mortality was higher than 1%. Brett (1998) suggested a similar threshold reporting that it was economically beneficial to vaccinate if the clinical incidence of OJD was at least 1% in breeding ewes provided that there was no deduction in monetary value of a carcass value due to vaccination.

In this study, we used stochastic simulation. In deterministic calculations all parameters have point average values that are assumed to be constant. Our estimation of OJD-specific mortality and economic outcomes were based on several biological parameters.

Inherently, such parameters are subject to biological and chance variation. Stochastic simulation took this into account by repeatedly sampling from parameter distributions thousands of times. The resulting posterior distribution of the benefit-cost of OJD vaccination therefore considered the biological variability of all input parameters to provide greater insight into the likelihood for a farm to achieve a positive financial return from vaccination, as illustrated in Figure 3.2.

In this study we used 20 farms known to have OJD and with owners or managers willing and able to participate and record the data required by the study design. This is therefore a potentially biased subset of the population, hence any inference beyond the farms and flocks included in the study may or may not be representative the population. By necessity, this study design was pragmatic since selecting a truly random subset of sheep farms in the population is not feasible when reliance on farmer choice to participate is an imperative. Nevertheless, the enrolled farms can be considered typical of fine-wool farms that view OJD as a problem. However, only a few farms with other breeds were included in the field studies, thus if anything, they might represent lamb producing farms with a comparatively high OJD incidence.

This study focused on economic loss due to both clinical and preclinical Johne's disease in ewes. Since infection with *Map* does not always progress to clinical disease in all sheep (Begg et al. 2017), it might be worthwhile to investigate the economic impact of subclinical paratuberculosis on ewe production traits such as growth, lamb production and wool quantity or quality. The economic impact of subclinical paratuberculosis in sheep is poorly documented and to the best of our knowledge only one previous study conducted in New Zealand investigated the effect of subclinical paratuberculosis on productivity in sheep (Thomson et al. 2002). That study found subclinical disease may not lead to significant production loss in sheep, but the results were based on study of only two farms. Thus, a longitudinal study incorporating a larger number of farms might help to better understand the impact of subclinical disease in sheep and evaluation of the economic effects.

### 3.5 Conclusions

This study presents estimates of mortality rates for OJD, its economic impact, and vaccination benefit-cost. It confirms that OJD mortality rate is higher in fine-wool breeds such as Merino than in meat breeds such as Romney. The incidence of OJD mortality in a flock was the most important determinant of economic cost and therefore benefit-cost of vaccination. Large variation was observed between farms and on some farms between years, hence the economic return of any intervention would also vary accordingly. The dependence on the clinical OJD incidence for economic return demonstrates that farmers need to tally OJD cases on-farm to inform decisions around adoption of vaccination, regardless of breed type.

The stochastic simulation addressed the state of OJD at equilibrium. At that stage, vaccination was cost-effective when the annual incidence of OJD mortality prior to vaccination was 1% or more. For fine-wool breeds, this provided a benefit-cost ratio of four, but this required a number of years of continued vaccination.

### 3.6 Acknowledgments

The research was primarily funded by the New Zealand Johne's Disease Research Consortium (JDRC), and partially co-funded by the New Zealand Sheep Industry Transformation Project, NZSTX, a Primary Growth Partnership Programme led by The New Zealand Merino Company and co-funded by the Ministry for Primary Industries New Zealand. We highly acknowledge the participating farmers and their vets for their co-operation, and JDRC and NZSTX for funding this study.

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## Intra-flock shedding and serum antibody ELISA positivity prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in non-clinical commercial sheep flocks in New Zealand

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**Abstract** – Paratuberculosis (PTb) is highly endemic in New Zealand livestock farms. We report the intra-flock prevalence of the causative agent of PTb, *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), shedding and serum antibody ELISA positivity estimated in 45 commercial sheep flocks in New Zealand. Faecal samples (n = 878) from non-clinical flocks with previously determined pooled faecal culture (PFC) status (n = 16 positive and n = 29 negative) were tested with direct faecal real-time quantitative PCR (qPCR) to determine *Map* shedding prevalence and abundance. Additionally, qPCR was compared with *Map* antibody ELISA results from 837 corresponding sera to correlate shedding prevalence with sero-prevalence.

Overall, 13.1% of faecal samples and 5.8% of serum samples tested positive. The median intra-flock prevalence (IFP) of *Map* shedding in the qPCR positive flocks was 13.5% (mean = 22.2%; 95% CI = 11.5–33%) with a range of 5–95%. Five samples (0.5%) in four of the flocks had a concentration of DNA  $\geq 1\%$  picogram/PCR and were classified as super-shedders. The mean DNA concentration in super-shedders was approximately 4,000 times higher than in other shedders. The median IFP of *Map* ELISA positivity in ELISA positive flocks was 10% (95% CI = 7.7–12.4%) with a range of 5–20%. ELISA sample-to-positive ratio and the DNA concentration in qPCR positive samples were positively correlated ( $r = 0.61$ ,  $p < 0.05$ ).

We conclude that 89% of the qPCR positive ewe flocks had a shedding prevalence range of 5–35%, with 11% having a prevalence  $> 80\%$ , and that about 1% sheep in qPCR positive flocks were super-shedders. The shedding status of flocks was more accurately assessed using a combination of qPCR and ELISA results on a selection of individual animals in the flock than by PFC of the same selection of samples.

**Key words:** *Mycobacterium avium paratuberculosis* (*Map*), qPCR, shedding, serum ELISA

**Authors:** Milan Gautam, Nelly Marquetoux, Marian Price-Carter, Anne Ridler, Peter R. Wilson, Cord Heuer

## 4.1 Introduction

Paratuberculosis (PTb) is a chronic debilitating intestinal disease of ruminants that results from infection with *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). Primarily, *Map* is transmitted by oral ingestion of the organism shed in the faeces of infected animals. Treatment of individual animals is not recommended or undertaken since there is no known effective treatment (Jean 1996). Infection with *Map* can be latent, characterised by colonisation of the host tissues without pathological signs, active, characterised by subclinical pathological signs, or clinical, also known as Johne's disease, which ultimately results in death (Marquetoux et al. 2018). A review by Whittington et al. (2017) provided a more comprehensive dichotomous classification of animals exposed to *Map*. The different stages of *Map* infection impact *Map* diagnostic test performance (Nielsen & Toft 2008). Only a small proportion of infected animals get to the clinical stage before they are removed from the flock for other reasons. Animals with subclinical infections may remain in the flock thereby playing a role in keeping flocks infected and maintaining infection (Marquetoux et al. 2018). This insidious shedding and spread of *Map* by non-clinical animals possesses a great challenge to PTb control and eradication efforts in ruminants (Chiodini et al. 1984).

Principally, control of PTb in domestic ruminants depends on early detection and removal of infectious animals. However, ante-mortem diagnosis of non-clinical PTb is often a difficult task as conventional diagnostic tests have limited sensitivity to diagnose such animals. Diagnostic tests for PTb in live animals are based either on detection of *Map* organisms or assessment of host immune response to *Map* infection. Isolation of *Map* from shedding animals by faecal culture has been considered as one of the gold standards for diagnosis of PTb due to specificity of nearly 100% (Whitlock et al. 2000). However, due to the slow growing nature of this pathogen, it can take several months to obtain results. Growth of *Map* in faecal culture is affected by strain type, contamination by other intestinal flora and load of viable organisms in the sample (Kawaji et al. 2011). Pooled faecal culture (PFC) is commonly used to determine flock-level infection status and this method is considered superior in terms of sensitivity and cost effectiveness to serological testing (Whittington et al. 2000a).

Identification of the IS900 element (Green et al. 1989), a multi-copy insertion element

present in the *Map* genome, provided the opportunity to develop sensitive PCR assays for rapid diagnosis of *Map* from clinical specimens (Vary et al. 1990). Besides their ascendancy over faecal culture in terms of having short turnaround time, PCR methods have been reported to have a sensitivity that is comparable to or higher than the radiometric faecal culture method (Bauman et al. 2016) and the assay can be designed to be highly specific for *Map* detection (Plain et al. 2014). Because of the high number of copies of the IS900 element in the *Map* genome, PCR assays based on this element are more sensitive than assays based on lower copy genes. While PCR tests are unable to differentiate viable *Map* cells from *Map* DNA *per se*, the above attributes, combined with ability to evaluate the degree of faecal shedding, make qPCR a useful tool for identification of highly infectious animals to target for removal from a flock and also for identifying low-risk replacement animals (O'Brien et al. 2013).

Super-shedding, a phenomenon described as a disproportionately high concentration of pathogens being contributed by a small fraction of infected contemporary individuals in a population is also observed in PTb (Slater et al. 2016). While the contribution of super-shedders in infection dynamics for PTb is not linear (Slater et al. 2016), early identification and removal of super-shedders is likely to be of significance in PTb control in infected flocks.

A previous survey estimated that at least 75% of ovine flocks in New Zealand were infected with *Map* (Verdugo et al. 2014a). In clinically affected flocks, the overall annual ewe mortality rate due to the clinical disease (OJD) was estimated at 1.8% in fine wool (Merino, Corriedale and Merino X) flocks and 0.68% in Romney flocks (Gautam et al. 2018a). In Australia the annual mortality due to OJD on affected farms ranged from 1.8 to 17.5% over three years (Bush et al. 2006). As for other infectious diseases, the intra-flock prevalence (IFP) would be a useful measure to evaluate the success of a PTb control program. Given the cost and time factor for faecal culture, it is not surprising that estimates of IFP are either not available (Nielsen & Toft 2009) or are often limited to sero-prevalence estimates [for example Rita et al. (2011), Stau et al. (2012)]. Sero-prevalence estimates are not always precise since the serologic tests for *Map* diagnosis have low sensitivity, particularly in non-clinical infections (Hope et al. 2000). Intermittent *Map* shedding by ewes without demonstrable clinical or serological signs may exist in infected flocks (Whittington et al. 2000b). These animals are less likely to be diagnosed

even by faecal culture if a single faecal sample is used (Whittington et al. 2000b). Intra-flock estimates of *Map* shedding and serum antibody ELISA positivity of *Map* prevalence are not available for New Zealand.

The objectives of this study were: a) to estimate IFP of *Map* shedding and exposure to *Map* in non-clinical commercial sheep flocks in New Zealand based on faecal PCR and serum ELISA respectively b) determine the proportion of shedders that are super-shedders, and c) analyse the relationship between *Map* shedding and serum antibody ELISA positivity, as measured serologically in non-clinical ewes.

## 4.2 Materials and methods

### 4.2.1 Collection of faecal and serum samples

The faecal and serum samples for this study were originally collected between June 2009 and July 2010 for a nationwide PTb prevalence survey on commercial sheep, beef cattle and deer farms (Verdugo et al. 2014a). Briefly, 9–20 animals per available species from 238 farms throughout New Zealand were randomly selected for paired blood and faecal sample collection. For flock-level testing of ovine flocks, a single pool of 20 ewes ( $\geq 2$  years of age) per flock was prepared for faecal culture. Faecal samples were cultured for *Map* using the BACTEC system at AgResearch, Infectious Diseases Laboratory, Wallaceville Animal Research Centre, Upper Hutt. Faecal material was first decontaminated using the double decontamination method described by Whitlock & Rosenberger (1990). Approximately 2 g of faeces was added to 40 ml of sterile distilled water, vigorously shaken and allowed to stand for 30 minutes. A 5 ml aliquot was taken off the top of the liquid and decontaminated in 1% cetylpyridinium chloride (CPC). After decontaminating at room temperature overnight the samples were centrifuged at 3,500 x g for 20 min at room temperature, the supernatants discarded and the pellets reconstituted in 1 ml of antibiotic cocktail [described in Whittington et al. (1998)]. After a further incubation at 37 °C for 3 days 0.5 ml was inoculated into a Bactec 12B vial, supplemented with the antibiotic cocktail PANTA (Becton Dickinson, Sparks, MD), 0.8 ml of sterile egg yolk, and mycobactin J (Allied Monitor, Fayette, MO). The inoculated vials were incubated at 37 °C for 12 weeks and read at weekly intervals for the presence of  $^{14}\text{CO}_2$ , and the

growth indices were recorded. Vials registering a positive growth index (GI >15) were examined for AFB using Ziehl-Neelsen (ZN) staining and also inoculated onto blood agar for the presence of microbial contamination. If a sample had a positive GI reading, but had negative ZN stain results, it was re-incubated until the end of 12 weeks before being tested again for the presence of AFB. If no AFB was detected at the end of 12 weeks, the sample was considered negative. *M. avium* subsp. *paratuberculosis* was identified on the basis of the presence of slow-growing clumps of AFB that were mycobactin dependent. Additionally, serum samples from ewes from PFC negative flocks were tested by ELISA. Unused individual faecal and serum samples were stored at -80°C for approximately eight years after collection.

For the present study, 878 individual faecal samples from the parent study (Verdugo et al. 2014a) were tested again using a direct faecal real-time qPCR assay. These samples came from 45 flocks (median = 20 individual ewes/flock, range = 9–20 ewes) of which 16 were previously determined as PFC positive with 318 individual samples and 29 flocks that were previously determined as PFC negative with 560 individual samples (Table 4.1). The selected flocks were from either sheep only (n = 9) or sheep and beef and/or deer (n = 36) farms. The 45 flocks were selected out of a total of 139 flocks whereas flocks that had one or more sheep suspected as being clinically affected with PTb (n = 23) at the time of sample collection in the parent study were excluded.

#### 4.2.2 Serum and ELISA test

For determining prevalence of *Map* serum antibody ELISA positivity, a total of 300 individual serum samples from 15 out of the 16 PFC positive flocks (serum samples from one PFC positive pool was missing) were thawed and tested in New Zealand Veterinary Pathology Limited using a *Map* antibody ELISA kit (paratuberculosis screening antibody test<sup>TM</sup>, IDEXX Laboratories). Results were combined with those from the 28 out of 29 PFC negative flocks (ELISA results from one PFC negative pool were missing) which were determined previously in the parent study (Verdugo et al. 2014a) in the same laboratory using a similar kit (then known as Pourquier<sup>TM</sup> ELISA, Institut Pourquier, Montpellier, France). ELISA results with sample-to-positive ratio (S/P) of  $\geq 55$  were considered positive.

### 4.2.3 qPCR test of faecal samples

Faecal samples stored in  $-80^{\circ}\text{C}$  were allowed to thaw at room temperature overnight before processing for qPCR. The multi-stage qPCR protocol involved recovery of *Map* cells from a faecal suspension, bead-beating for cell disruption and DNA extraction using magnetic beads followed by semi-quantitative qPCR. PCR was run in duplicate on each sample.

#### Recovery of *Map* cells from faecal suspension and bead-beating

Faecal suspensions were prepared by following the low-input workflow described in the MagMAX<sup>TM</sup> core mechanical lysis module user guide (Thermo Fisher Scientific)<sup>1</sup>. Briefly, we added 0.34 g (range 0.3–0.4 g) of faecal sample followed by addition of 1 ml sterile milliQ water into 2 ml sterile tubes. Samples were suspended by vortexing vigorously for three minutes. The suspension was then centrifuged at 100 x g for 30 sec. 175  $\mu\text{L}$  of the faecal supernatant was transferred to a bead beating tube (MagMAX<sup>TM</sup>) containing zircona beads and 400  $\mu\text{L}$  clarifying solution (MagMAX<sup>TM</sup>). Samples were disrupted in a mechanical cell disrupter (TissueLyser II<sup>TM</sup>, Qiagen) set to the maximum speed of 6.5 m/sec for 60 seconds, twice. The bead tubes were then centrifuged at 15,000 x g for three minutes.

#### DNA extraction

The supernatants containing DNA were washed as indicated in the MagMAX<sup>TM</sup> core mechanical lysis module user guide (Thermo Fisher Scientific), and then eluted using a magnetic particle processor (KingFisher<sup>TM</sup> Flex; Thermo Fisher Scientific). The eluted DNA was diluted five-fold to reduce PCR inhibition (Acharya et al. 2017). Both the undiluted and diluted DNA extracts were stored at  $-20^{\circ}\text{C}$  before being tested by qPCR.

#### IS900 quantitative PCR

Quantitative PCR of the DNA extracts was performed in a real-time PCR machine (Rotor-Gene Q<sup>TM</sup> 5PLEX, Qiagen) as previously described by Plain et al. (2014). The final

<sup>1</sup><https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0015945-MagMAXCORE-Mech-Lysis-Module-UG.pdf>

volume of reaction mixtures contained 20  $\mu\text{L}$  mastermix (250 nM final concentration of each forward [MP10-1 (5'-ATGCGCCACGACTTGCAGCCT-3')] and reverse [MP11-1 (5'-GGCACGGCTCTTGTTGTAGTCG-3')] primers; SensiMix<sup>TM</sup> (SYBR LOW-ROX qPCR mastermix; Bioline; and milliQ water) and 5  $\mu\text{L}$  diluted DNA extract loaded into a 100-well PCR ring (Rotor-Disc<sup>TM</sup> 100; Qiagen). The following amplification settings were used: initial denaturation at 95°C for 9.5 min; 45 cycles of denaturation at 95°C for 30 s, annealing at 68°C for 60 s, and melt curve analysis from 65–97°C.

#### *Controls and acceptance criteria for qPCR results*

For each batch, three controls were taken through the preparation process from the faecal sample step to the IS900 qPCR.

- (a) positive control: faecal samples were collected from New Zealand ewe lambs which were experimentally infected with *Map* (Smith 2016). These lambs had histopathological lesions of PTb in the intestines and the faecal samples were acid fast bacilli positive.
- (b) negative control: faecal samples from a sheep from a non-infected flock (repeat negative tests at the flock level and history of freedom from *Map* infection) and determined to be faecal culture negative. This sample was imported from the University of Sydney, Australia.
- (c) process control: all reagents, but without faecal sample.

In every qPCR run, a standard curve of *Map* genomic DNA (Telford 9.2 strain) serially diluted from 10 to 0.001 pg/reaction was included. Each qPCR run also included no-template controls (5  $\mu\text{L}$  purified sterile water mixed with all PCR reagents). Two replicates of each individual sample including the controls and the standards were tested by qPCR except for standard 5 (0.001 pg/reaction), which had four replicates. The following combinations of acceptance criteria based on Plain et al. (2014) were used for accepting the qPCR step.

- (a) PCR amplification efficiency of the *Map* genomic DNA standard curve, including both replicates of standard 1–4 and at least two replicates of standard 5, between 90 and 110%.

- (b) both replicates of no template qPCR control negative
- (c) dissociation peak ( $T_m$ ) in melt curve analysis for positive controls, standards and samples between 87.6–90.6°C.

The entire process of a faecal test was accepted if both replicates of the process control and the negative faecal control were negative and the positive faecal control replicates were positive, in addition to an acceptable qPCR run. For all acceptable qPCR runs, a faecal sample was considered positive if at least one of its two replicates had the correct dissociation peak ( $T_m$ ) and DNA concentration of  $\geq 0.001$  pg/reaction. The decision criteria used for DNA quantification of positive samples is outlined in Figure 4.1. Super-shedders were identified based on the DNA quantity in the tested samples.

#### 4.2.4 Statistical tests

Results were analysed in R (version 3.5.0) (R Development Core Team 2015) and Microsoft packages (MS Excel and Access 2010). Descriptive analyses were performed including means, medians, quartiles, histograms, and box, whiskers and scatter plots and 95% confidence envelopes. The relationship between PCR and serology was analysed using simple linear regression.

### 4.3 Results

Overall 13.1% of the faecal samples were qPCR positive ( $n = 115$ , Table 4.1). In the PFC positive flocks, at least one faecal sample was qPCR positive in 81.2% (13/16) versus 44.8% (13/29) in the PFC negative flocks. 89% of the qPCR positive ewe flocks had a shedding prevalence range of 5–35%, with 11% having a prevalence  $>80\%$ .

Overall 5% of serum samples tested ELISA positive ( $n = 42$ ) including 3.8% ( $n = 28$ ) from the qPCR negative ewes. At least one serum sample was ELISA positive in 78.5% ( $n = 11$ ) PFC positive and 46.4% ( $n = 13$ ) PFC negative flocks.

The median IFP of shedding in qPCR positive flocks was 13.5% (mean = 22.2%; 95% CI = 11.5–33%; range 5–95%), whereas the median IFP of serum antibody ELISA positivity

in the serum ELISA positive flocks was 10% (mean = 10%; 95% CI = 7.7–12.%; range 5–20%).

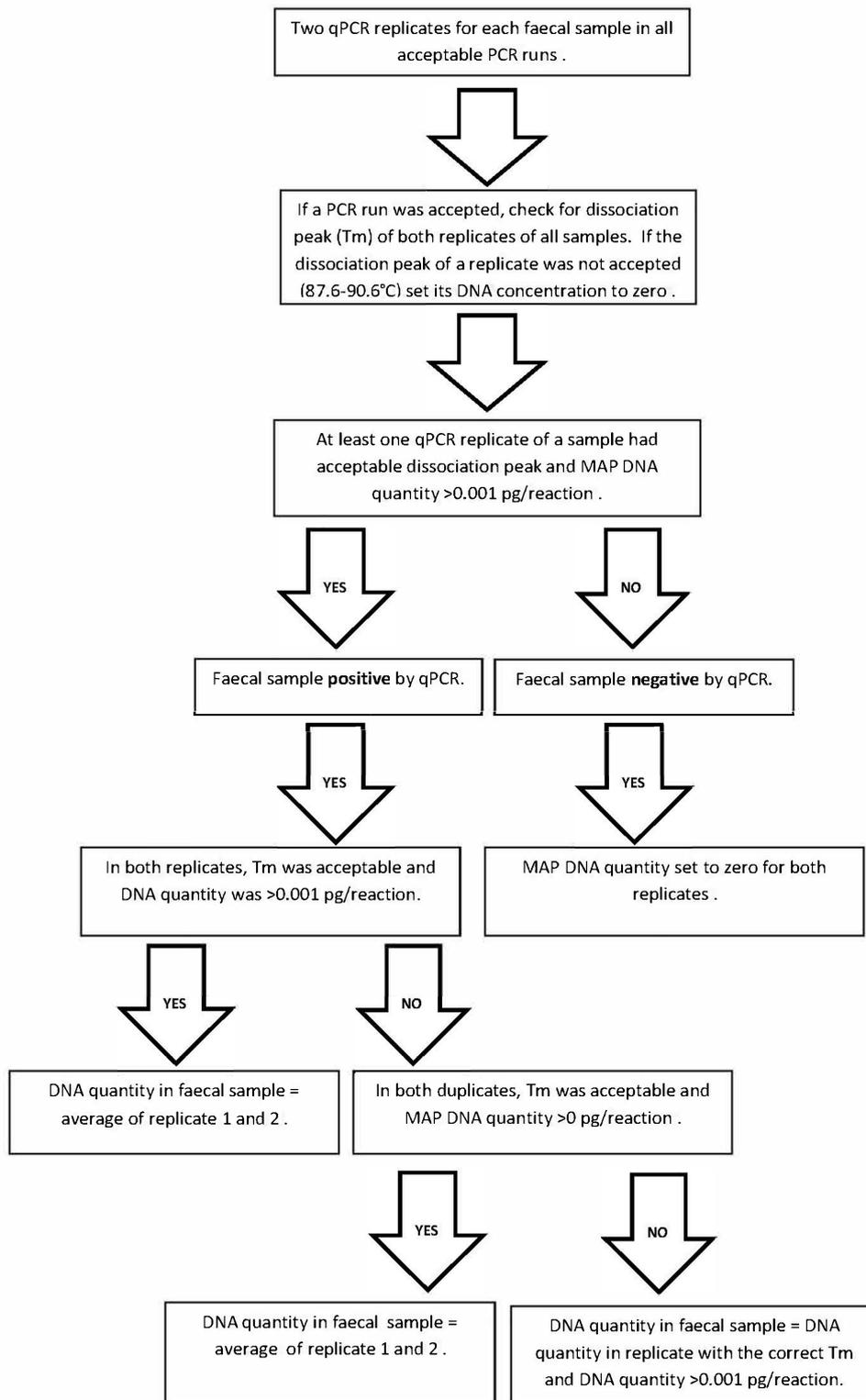
The distribution of DNA concentrations in qPCR positive faecal samples was right skewed as shown in Figure 4.2. Figure 4.3 presents boxplots of DNA quantity in qPCR positive faecal samples categorised by PFC status. Five ewes in the tail of the DNA distribution (Figure 4.2) had disproportionately high DNA quantity in their faeces and were thus classified as super-shedders. Three PFC positive flocks had one super-shedder each while one PFC negative flock contained two super-shedders. These super-shedders shed *Map* quantities over three orders of magnitude higher than other ewes in the same flock, and the mean DNA concentration in super-shedders and other shedders was 30.5 pg/reaction (95% CI = 8.47–69.5) and 0.0077 pg/reaction (95% CI = 0.00217–0.0133), respectively. The distribution of DNA concentration in shedders and super-shedders is shown in Figure 4.4.

All super-shedders tested positive by ELISA and a linear relationship between ELISA S/P and DNA concentration ( $r = 0.61$ ;  $p < 0.05$ ) was observed (Figure 4.5).

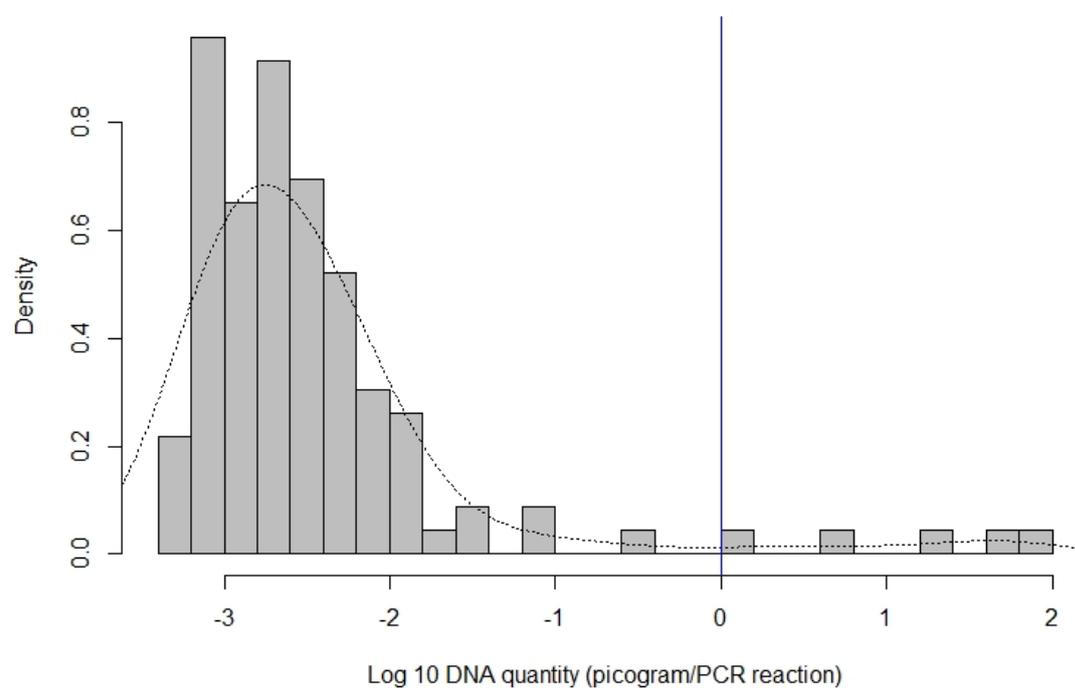
**Table 4.1:** Number of samples tested and number positive to direct faecal quantitative PCR (qPCR) and serum ELISA for sheep flocks previously determined as positive and negative using pooled faecal culture (PFC)

PFC status	Farm type	qPCR of faecal samples			Serum ELISA		
		Positive	Total tested	Prevalence (%)	Positive	Total tested	Prevalence(%)
negative	SB	0	17	0	0	17	0
	S	0	17	0	0	17	0
	S	0	20	0	0	20	0
	SD	0	9	0	0	9	0
	SB	0	20	0	0	20	0
	SD	0	20	0	0	20	0
	SB	0	20	0	0	20	0
	SD	0	20	0	1	20	5
	SD	0	20	0	1	20	5
	SD	0	20	0	1	20	5
	SD	0	20	0	1	20	5
	SB	0	20	0	1	20	5
	S	0	20	0	2	20	10
	S	0	20	0	2	20	10
	SBD	0	20	0	3	19	16
	S	0	20	0	4	20	20
	S	1	20	5	0	20	0
	SD	1	20	5	1	20	5
	SD	1	20	5	2	20	10
	SBD	2	20	10	0	20	0
	SB	2	20	10	0	20	0
	S	2	17	12	0	17	0
	SB	3	20	15	0	20	0
SBD	3	20	15	0	20	0	
SBD	3	20	15	0	20	0	
SB	5	20	25	2	20	10	
SD	5	20	25	3	20	15	
SB	7	20	35	0	20	0	
SB	18	20	90	Na	Na	Na	
positive	SB	0	20	0	0	20	0
	SB	0	20	0	0	20	0
	SBD	0	20	0	1	20	5
	SB	1	20	5	0	20	0
	SB	1	20	5	0	20	0
	SBD	1	20	5	1	20	5
	SB	1	20	5	1	20	5
	SB	1	20	5	Na	Na	Na
	SBD	1	19	5	2	19	11
	SB	2	20	10	4	20	20
	SB	3	20	15	1	20	5
	SBD	3	20	15	3	20	15
	SBD	5	19	26	1	19	5
	S	7	20	35	3	20	15
	S	17	20	85	3	20	15
	SB	19	20	95	4	20	20

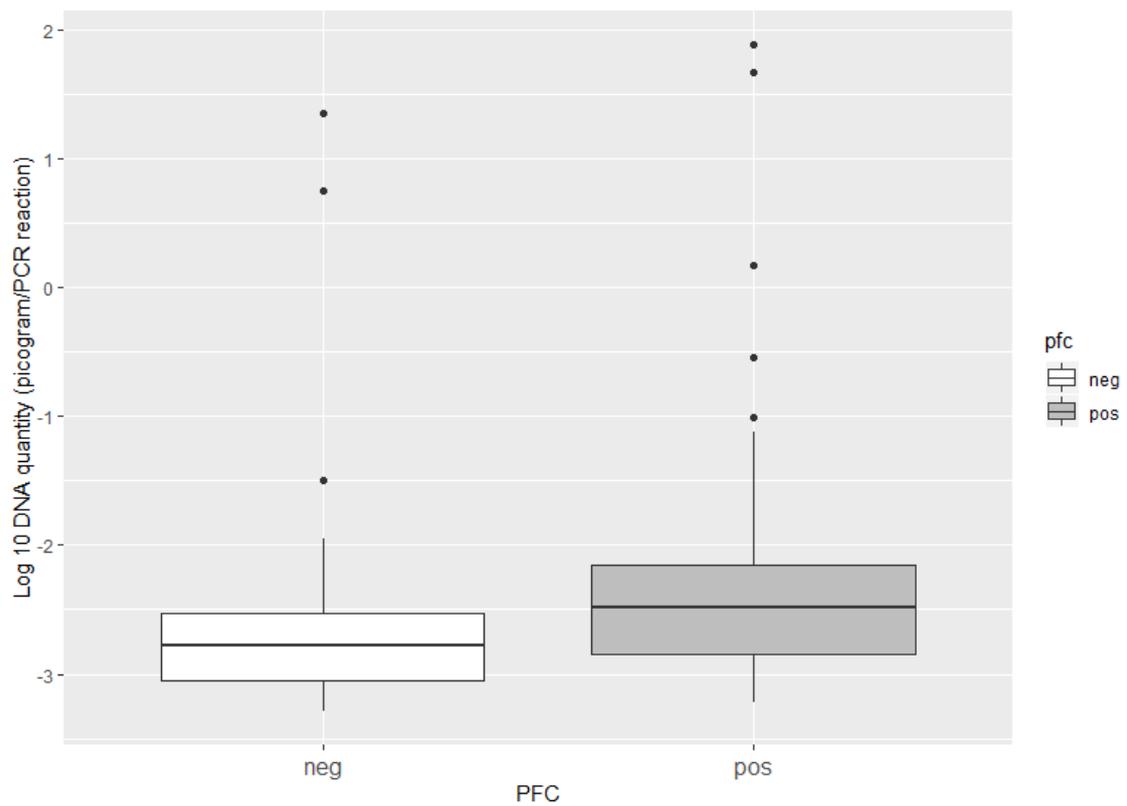
S = sheep; B =beef; D = deer Na = Not applicable



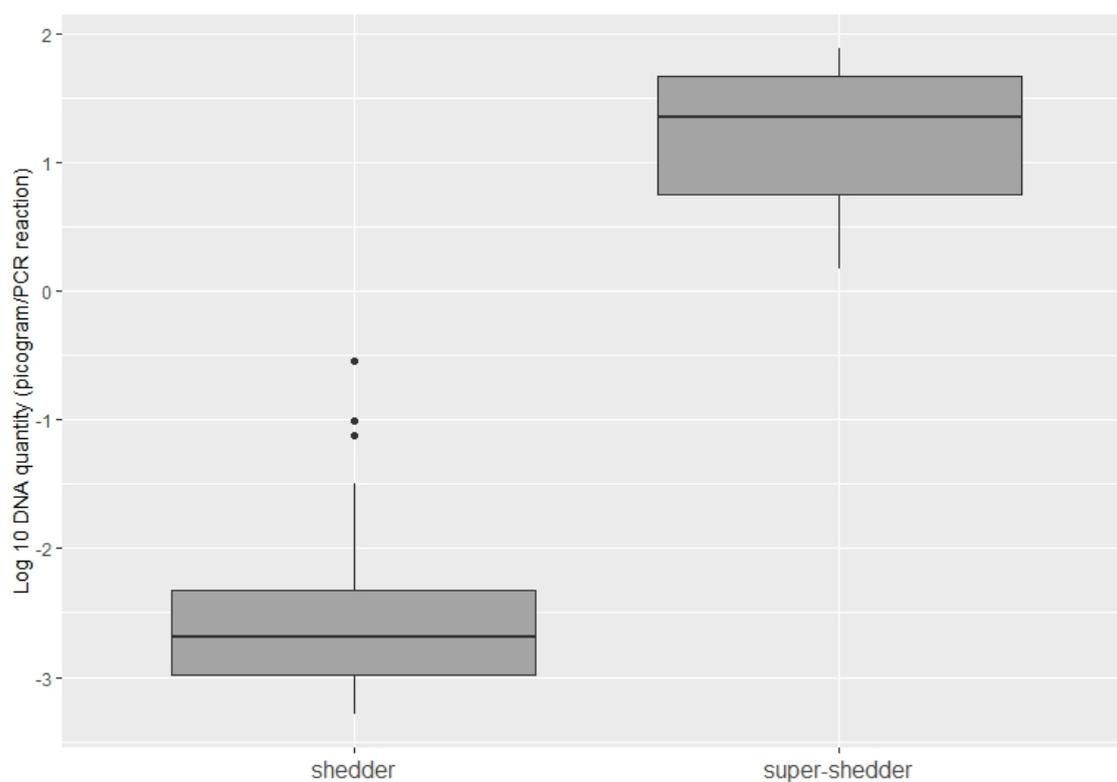
**Figure 4.1:** Flowchart of the decision criteria used to categorise and quantify qPCR results of faecal samples.



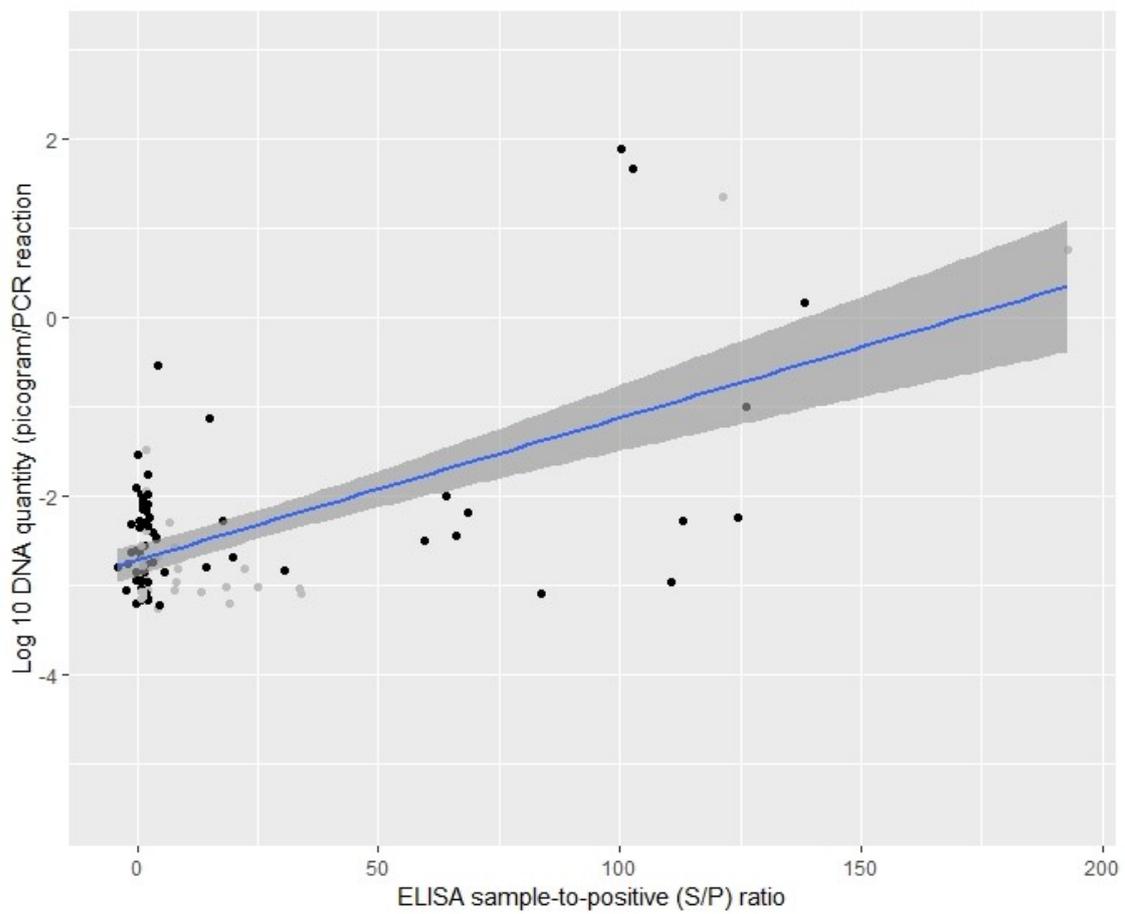
**Figure 4.2:** Density histogram of log<sub>10</sub> DNA quantity in qPCR positive faecal samples. Samples to the right of the vertical line on the X-axis represent super-shedders. In this case, vertical axis is measured as a probability density of log<sub>10</sub> DNA quantity and the total area under the curve (dotted) is equal to 1.



**Figure 4.3:** Boxplot of log<sub>10</sub> DNA quantity in qPCR positive faecal samples from flocks categorised by PFC status as negative (neg) and positive (pos). Observations designated as outliers are plotted as separate points.



**Figure 4.4:** Boxplot of average quantity of DNA based on qPCR by shedding status in qPCR positive samples. Observations designated as outliers are plotted as separate points.



**Figure 4.5:** Scatterplot and trend line of the relationship between ELISA S/P and DNA concentration (log<sub>10</sub>-picogram/PCR reaction) of qPCR positive ewes from both PFC positive (black) and negative (grey) flocks. The shaded region represents the 95% confidence intervals.

## 4.4 Discussion

We present the first estimate of IFP of both *Map* shedding serum ELISA *Map* positivity in non-clinical adult ewes in 45 purposively selected New Zealand commercial flocks. We tested a subset of individual animal samples, which were originally collected in a different survey and PFC status had been assigned to the flocks based on a single pool of 20 random samples per flock. In PFC positive flocks, 81% had at least one and up to 19 of 20 samples that were qPCR positive. Because of the extra sensitivity provided by qPCR of individual animals, 44% of flocks that were PFC negative had between one and 18 qPCR positive sample. Five qPCR positive sheep were super-shedders with one each from three PFC positive flocks and two from one PFC negative flock. Approximately 79% of PFC positive flocks and 46% of PFC negative flocks had at least one sheep which tested positive by serum ELISA. Faecal qPCR and serum ELISA S/P were positively correlated. Estimates of IFP presented here represent apparent rather than true prevalence of PTb. We recognize that our interpretation of the intra-flock shedding and exposure prevalence is based on imperfect testing methodology since neither IS900 PCR nor serum ELISA are perfect for PTb diagnostics (Mathevon et al. 2017). Nevertheless, at the moment these are the best techniques available to access *Map* shedding and exposure status in New Zealand sheep flocks. Bayesian methods have been used elsewhere to determine the ‘true prevalence’ of PTb in infected flocks based on pooled faecal culture and ELISA (Verdugo et al. 2014a). We did not use this approach here because the aim of the study was to estimate intra-flock shedding and exposure prevalence with the proviso that our estimate, while being subject to imperfect sensitivity and to a probable lesser extent specificity limitations, describes shedding and serum antibody ELISA positivity at a detectable level, which is likely to be of practical significance.

Furthermore, *Map* infection initially elicits a cell-mediated immune response in the host followed by humoral immunity as the disease progresses (Chiodini et al. 1984). PTb pathophysiology also involves intermittent (Mitchell et al. 2015) and passive *Map* shedding (Kralik et al. 2014) and the role of these immune responses in faecal shedding patterns at different stages of disease progression are not well understood (Magombedze et al. 2016). Current diagnostic tests cannot perfectly identify and quantify *Map* and associated humoral response in such scenarios. In order to better understand the utility of diagnos-

tic tests, a longitudinal study design to repeatedly test animals until they develop clinical signs or are culled for reasons other than PTb might be preferred to cross-sectional studies (Beaver et al. 2017). Examples of overseas studies with longitudinal study design in which animals were repeatedly tested are available (Rast & Whittington 2005, Schukken et al. 2015).

Among the 29 PFC negative flocks, 45% had at least one animal that tested qPCR positive, 46% had one or more ELISA positives and 68% had either a qPCR or an ELISA positive animal. The respective proportions for the 16 PFC positive flocks were 81%, 73% and 88%. We observed that combination of qPCR and ELISA of multiple individual samples from a flock had a greater utility for determining the flock-status, in terms of shedding, than PFC reported earlier by Whittington et al. (2000a) and Verdugo et al. (2014a) in Australia. However, it is noted that the reported flock level sensitivity of PFC in Verdugo et al. (2014a) was 50% compared to a much higher sensitivity of 90% reported by Sergeant et al. (2002) in an Australian study. This difference in sensitivity might have partly resulted due to reasons discussed later in the text.

The IFP of shedding and serum antibody ELISA positivity reported here is in adult ewes in flocks with zero to low annual incidence of clinically affected animals, as reported by Verdugo et al. (2014a). Based on the presence of histological lesions, prevalence of PTb in six (of which four were known be clinical PTb positive) Merino flocks in Australia was estimated within a range of 9–22% (Sergeant et al. 2003). Other overseas estimates of intra-flock prevalence were all based on serology (ELISA), thus they describe only exposure rather than shedding. For example, Stau et al. (2012) report a mean IFP of 21% (sd 13%) based on ELISA for 1,473 individual animals from 150 flocks in Germany. In contrast to our study, their study sampled sheep in low body condition so the sample populations are not comparable. Since *Map* antibody detection tests such as ELISA are relatively inexpensive and easier to perform compared with tests that detect *Map* antigen, the former are often used to determine prevalence of exposure. However, ELISA sensitivity is generally low, particularly if an animal is not clinically affected. Consequently, such studies tend to underestimate the overall exposure status.

Commercial PCR testing of ovine faecal samples is currently not a widely used method for PTb diagnosis in New Zealand, most likely due to a lack of economic or control imperatives. A Sub-committee on Animal Health Laboratory Standards (SCAHLs, now

dissolved)-validated PCR protocol (Plain et al. 2014) for cattle and ovine faecal sample processing, DNA extraction and qPCR is available. At the inception of this study, we intended to follow the same protocol for testing our ovine faecal samples. However, at the time we tested the samples, a new sensitive and simpler to perform *Map*-specific kit for processing and DNA extraction from bovine and faecal samples became available (Thermo Fisher Scientific). We therefore ran an internal validation study on samples processed by this new method and characterised by the Plain et al. (2014) PCR protocol. This was done by in-house 'spiked' faeces experiments in which *Map*-free faecal samples were spiked with suspensions consisting of a known number of Telford 9.2 strain *Map* cells. We observed that the PCR results from both the original and the newly available methods for faecal sample processing and DNA extraction were comparable. These data were not reported in this study. Working with the latter method to process samples and extract DNA involved a lesser volume of faecal sample and processing time as well as fewer manual steps. Given that hundreds of faecal samples were tested in the present study, the sample processing time advantage of the newer method was considerable and hence we adapted it for processing of faecal samples and DNA extraction in our study. The qPCR part of the original protocol (Plain et al. 2014) was kept intact.

We could not achieve the lower limit of detection (10 *Map* cells/g of faeces) that was reported by Plain et al. (2014) regardless of the kit used. In our case, the lower limit of detection was 400 *Map* cells/g of faeces. While we attempted to follow the original protocol and use the same equipment throughout the entire process as closely as possible, variations in the personnel involved in the faecal testing and unavoidable differences in some of the equipment might partly explain the disparity between these results. We acknowledge that a lower limit of detection for the qPCR method would have been desirable to increase the apparent sensitivity of the qPCR test. In our spiked faeces qPCR experiments we observed that DNA concentration (pg/reaction of the PCR run) estimated by qPCR increased linearly as the concentration of *Map* cells in suspensions used for spiking faecal samples increased. However, as the primer in our PCR amplified the multicopy IS900 sequence in the *Map* genome that could be present in variable numbers in different genomes, estimates of DNA concentration from the PCR runs could not be used to estimate the corresponding number of *Map* cells in the starting material, which in our case was faecal samples. Our qPCR results were thus semi-quantitative and we interpreted

them accordingly.

Faecal and serum samples sampled in this study were from a subset of samples collected by a previous study (Verdugo et al. 2014a), which was one of the largest postal surveys of commercial sheep flocks, in terms of the number of farms involved, in New Zealand. This repository of faecal samples was representative of commercial sheep flocks that most likely viewed *Map* infection as a potential problem in their farms and therefore consented to participate in the survey. Farms with no history of *Map* infection were less likely to have participated in the study. In this sense, participating sheep farms may have different *Map* shedding patterns compared to the general population of all sheep farms in the country. Additionally, individual animals tested by qPCR and serum tests in our study were from only those flocks in which clinical suspect animals were not present at the time of sampling in the Verdugo et al. (2014a) study. Hence our estimates of faecal *Map* shedding and antibody in serum are more representative of the non-clinical, but *Map* infected commercial sheep flocks in New Zealand. It is likely that if individual animals from flocks, in which clinical suspect ewes were present, were tested by qPCR, the median prevalence of *Map* shedding might have been higher than that was observed in our study.

In our study, previously-determined PFC underestimated flock-level infection and shedding since almost half of the PFC negative flocks tested positive by qPCR and serum ELISA, when individual animals rather than pools of faecal samples were tested. These results have several potential explanations. Firstly, the PFC status of flocks was based on faecal culture described earlier (Verdugo et al. 2014a) in which a lower PFC sensitivity was reported than other overseas studies (Whittington et al. 2000a, Sergeant et al. 2002). It was noted that the pool size comprised of 50 individual animals in the Whittington et al. (2000a) study compared to a lower pool size of 9–20 individual animals in the Verdugo et al. (2014a) study. Furthermore, the number of pools per flock in the Whittington et al. (2000a) study ranged 1–11 compared to just one in the Verdugo et al. (2014a) study. While pool cultures were also tested by IS900 PCR by Whittington et al. (2000a), no such testing of pool cultures was conducted in the Verdugo et al. (2014a) study. Additional reasons for difference in results between these two studies may include difference in contamination rate between the two different labs or the distribution of the multi- or paucibacillary cases in sampled flocks (Whittington et al. 2000a). Apart from that, while the culture method utilised was developed to grow the sheep strain (Type S1), this strain,

in general, grows more slowly and less efficiently than the cattle (Type C) strain (Collins et al. 1990), whereas the qPCR likely detects DNA from both strains equally (Plain et al. 2014). This attribute of qPCR also raises the question whether pooled faecal PCR would be more sensitive than PFC for detection of *Map* in sheep faeces. Pooled faecal PCR is not a commonly used method for PTb screening although the technique has been reported in a spiked faeces experiment by Mita et al. (2016), in which *Map* DNA was detected in pools of 10 or fewer samples without loss of sensitivity. There are no reports available of pooled faecal sample qPCR in naturally infected sheep. A parallel comparison between the PFC and qPCR on the same set of fresh pooled faecal samples might be worthwhile to determine the utility of these methods for routine flock screening. Secondly, faecal culture requires viable *Map* cells whereas PCR detects DNA from both viable and non-viable cells. Finally, *Map* cells clump extensively (Grant et al. 2003), which poses a challenge in mixing pooled faecal samples homogeneously and might affect the outcome of culture. Despite these preferential features of PCR over faecal culture, some caution must be exercised when interpreting quantitative data based on qPCR in relation to *Map* shedding since passive passage of *Map* is possible in a heavily contaminated farm environment (Marquetoux et al. 2018). Additionally qPCR of individual animal samples is more expensive than PFC (Mita et al. 2016), which might influence the usefulness of the qPCR test for flock screening.

Super-shedders had approximately 4,000 times more DNA in faecal samples than other shedders when using the definition criteria chosen here. It is notable that two super-shedders were detected in a flock that was PFC negative. One was observed in each of three PFC positive flocks. The phenomenon of super-shedding in PTb is accepted (Pradhan et al. 2011, Slater et al. 2016), but a universal definition of super-shedders has not been agreed. The terminology commonly denotes contemporary animals excreting disproportionately high numbers of *Map*. In cattle, a super-shedder may produce up to 10,000 times as many *Map* cells/g (Slater et al. 2016) as others. The impact of super shedders on the epidemiology and pathophysiology of PTb in ovine flocks remains unclear. A simulation study of *Map* in cattle reported the lack of a linear, but rather a concave relationship between bacterial load and the *Map* infectivity (Slater et al. 2016). That study reported that for every 1,000-fold increase in the number of *Map* shed, the estimated increase in infectiousness was 2–3 fold. Based on our results, flocks in which the supershedders were

detected were not necessarily the ones with highest intraflock prevalence of *Map* shedding and therefore, it is likely that their role in *Map* flock infectivity is clear. Nevertheless, super-shedders remain a factor to consider for *Map* environmental contamination (Pillars et al. 2009) and are commonly targeted for PTb control programmes in other species (O'Brien et al. 2013). Further studies should be undertaken to establish the exact role of *Map* supershedders for flock infection in sheep.

We observed a positive linear correlation between serum ELISA S/P and qPCR derived DNA quantity in faecal samples. This trend concurs with results of a dairy cattle study (Beaver et al. 2017) in which spikes in faecal shedding corresponded with subsequent positive milk ELISA results, and in deer (O'Brien et al. 2013) in which positive predictive values for significant faecal shedding were 0.55 and 0.89 for serum ELISA unit cut-off of  $>50$  and  $\geq 150$  respectively, where high ELISA S/P was indicative of shedding.

In this study, all five ewes identified as super-shedders were also positive by ELISA, albeit not with the highest ELISA S/P values. Had these individual animals been cultured they are also likely to have been positive. Based on these results, serum ELISA might be useful to predict super-shedding individual animals among a flock of clinically normal adult ewes. A practical approach for farm managers might be to target ELISA positive individual ewes for qPCR test (Aly et al. 2012), rank results by S/P and DNA quantity, and prioritize removal of the highest probable shedders before the onset of clinical disease. Likely in this testing strategy low shedders might be subjected to continued testing once or twice a year.

Here we used an IS900 based PCR assay. IS900, a multicopy element in *Map* genome, which is an extensively used and well established molecular target in antigen-based *Map* diagnosis (Plain et al. 2014, Mathevon et al. 2017). Although there are reports in the literature of other organisms that harbour IS900-like sequences in their genomes that could potentially lead to false positives (Cousins et al. 1999, Englund et al. 2002), the IS900 assay developed by Plain et al. (2014) had been designed to avoid this type of non-specific detection. A different genetic element, F57, in the *Map* genome with no known similarities to other related organisms, could be screened by PCR in place of IS900 in order to increase the specificity of the PCR test (Sidoti et al. 2011). However, F57 is a single copy element and PCR techniques that target it have lower sensitivity than those targeting a multicopy genomic fragment such as IS900 (Castellanos et al. 2012).

There are several factors that negatively affect the accuracy of the qPCR test. In this study, we defined faecal samples with at least one PCR replicate positive as a positive sample. Faecal samples with one negative PCR replicate had, on average, lower DNA concentrations, suggesting a smaller number of *Map* organisms were being shed at the time when the sample was collected. This lower shedding level was at the detection limit by this qPCR, explaining most discrepancies between the two qPCR replicates and supporting the assertion that the host was actually infected. There is no way to distinguish low level shedding from actively infected animals from passive shedding from animals that ingested *Map* and shed it without the bacteria passing the gut wall and infecting the host. Passive shedders may be expected to shed lower numbers of *Map* in their faeces than animals with active infections (Pradhan et al. 2011). If actively infected sheep shed *Map* below the level of detection by qPCR, our IFP estimate would be lower than the true shedding prevalence. Accurate identification of infected animals, particularly those with low levels of DNA in their faeces may be achieved in principle by repeated sampling (van Schaik et al. 2003), although this approach might have limited practical applicability, particularly in large flocks, due to increased cost. Inferences about shedding from qPCR would be more accurate if a clearer distinction was possible between levels of normal shedding and mere passive shedding. Because qPCR is more frequently being used as a routine test for PTb diagnosis, it would be desirable to be able to differentiate passive transfer from other forms of true *Map* infection (O'Brien et al. 2013).

The faecal and serum samples tested for this work were stored at  $-80^{\circ}\text{C}$  for approximately eight years after collection pending the development of improved diagnostic tests, and financial resources. In a study of *Map* survival in faecal samples from naturally infected cattle stored at  $-18^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$  for up to 28 months measured by faecal culture, the odds of reduction in *Map* viability over time was statistically significant for samples stored at  $-18^{\circ}\text{C}$ , but non-significant for samples stored at  $-70^{\circ}\text{C}$  (Raizman et al. 2011). Storage of faecal samples at  $-80^{\circ}\text{C}$  yielded a higher quantity of DNA than from those stored at  $-20^{\circ}\text{C}$  from both sheep and cattle (Plain et al. 2014), hence the quantity of DNA detected by qPCR in this study should not have been greatly affected by storage.

## 4.5 Conclusions

Intra-flock prevalence of shedding in these low-zero clinical incidence flocks ranged from one to 90 or 95% in PFC negative and positive flocks, respectively, though almost twice the proportion of PFC positive flocks had  $\geq 1$  qPCR positive than PFC negative flocks and 44% of previously PFC negative flocks had  $\geq 1$  individual qPCR positive ewe. No animals were qPCR positive in almost 20% of previously PFC positive flocks. Results support that a combination of diagnostic methods increases the reliability determination of flock status. Overall, 1% of shedders in the sample sets from these flocks were super-shedders and 13% of flocks contained  $\geq 1$  shedder. Either one or two super shedders were observed in sample sets from 9% of the tested flocks, with most in PFC positive flocks. The IFP of shedding, determined by qPCR, was higher than the IFP of serum antibody ELISA positivity. There was a greater likelihood of ewes with a high S/P ratio being high shedders supporting ELISA as a screen test for targeted qPCR to predict high shedders. These data will aid informed decision making for PTb control on-farm and inform future research.

## 4.6 Acknowledgements

This work is a part of the PhD project of the first author who is funded by New Zealand International Doctoral Research Scholarship (NZIDRS) for his study. Technical support by Neville Haack in qPCR testing of faecal samples and Kristene Geyde in interpretation of PCR results is highly acknowledged. Positive DNA controls were obtained from Stefan Smith's PhD project and faecal samples for PCR negative controls from University of Sydney, Australia.



**Molecular epidemiology of Type S1  
*Mycobacterium avium* subspecies  
*paratuberculosis* isolates in New Zealand and  
Australian Telford strain based on single  
nucleotide variant analyses of whole genome  
sequence data**

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**Abstract** – Infection with *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) causes paratuberculosis (PTb), which is highly endemic on all major New Zealand livestock farms including those with sheep. Improved characterization of the *Map* pathogen and a deeper understanding of its epidemiological and molecular features can potentially inform about sources of infection, host-specificity, transmission pathways, geographic distribution and possible differences in pathogenicity of various strains. Such an understanding will be useful for improving current PTb control tools or development of new ones.

Perhaps because they originate from a restricted geographic area with a limited diversity of imported *Map* types, traditional *Map* genotypic methods have limited discriminatory power to distinguish New Zealand and Australia Type S1 genomes. There is only limited information about genomes of Type S1 *Map* from these countries using the ultimate resolution provided by whole genome sequencing (WGS). In the present study, 56 Type S1 *Map* isolates from New Zealand and the Type S1 Telford *Map* strain from Australia were characterized based on single nucleotide variant (SNV) analyses of WGS data by mapping them against Telford1, a closed genome of the Telford 9.2 strain (National Center for Biotechnology Information, reference sequence: CP033688, Brauning et al. (2019)). The relationship between the New Zealand *Map* genotypes, the possible origin of strains ‘virulence indicators’ (comprising histopathological categories of intestinal lesions, serology and diagnosis by vets after necropsy) putatively linked to the phenotypic expression of PTb severity in individual animals infected with *Map* was also evaluated.

Genotypes of the Type S1 *Map* isolates from New Zealand and the Telford isolate from Australia formed a closely related group, but they were quite distinct from the Type S1 and S3 isolates from other countries in Europe. Within New Zealand, *Map* genotype and region of sheep farm location were significantly associated ( $p < 0.05$ ). In contrast, we did not observe any association between genotype and the ‘virulence indicators’ putatively linked to severity of *Map* infection in infected animals. Our results of WGS-based SNV comparisons of *Map* isolates indicate the role of *Map* genotype in the observed difference in phenotypic expression of PTb in *Map* infected sheep is probably limited.

**Key words:** *Mycobacterium avium* subspecies *paratuberculosis* (*Map*), Type S1, whole genome sequence; single nucleotide variants (SNV)

**Authors:** Milan Gautam, Marian Price-Carter, Patrick Biggs, Petra Möbius, Cord Heuer

## 5.1 Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (*Map*) is the causative agent of paratuberculosis (PTb). It is an intracellular, gram positive, acid fast bacillus (AFB) of which two major subtypes (Type S and C) exist. With the advent of more advanced genotyping methods the nomenclature and classification of the different types has changed. The most recent phylogenetic classification based on whole genome sequencing (WGS) data is shown in Figure 5.1. In the rest of this chapter we will use the designation ‘Type S1’ to denote to the Type S subtype I *Map* isolates. Typically, PTb has a prolonged incubation period and in sheep the clinical disease is commonly observed in animals older than two years (Whittington & Sergeant 2001). The clinical disease expresses as a chronic enteropathy leading to severe loss of the body condition and eventual death of affected

animals. While *Map* most commonly infects ruminants and host preferences may be observed for different strains (Verdugo et al. 2014a), the organism is not host specific (Whittington et al. 2017). Some of the observed host preferences may be for reasons other than affinity for the host; for instance most New Zealand deer, but few New Zealand cattle carry a certain Type C that is prevalent in cattle in other parts of the world (Sonawane et al. 2016). This observed difference is likely to be due to the source of this introduction of infection into New Zealand rather than a preference for deer over cattle hosts. Published evidence of differences in virulence within a single type is limited (Stevenson 2015). In the present study, we focus on Type S1 *Map*, which included a group of field isolates from New Zealand with variable phenotypic ‘virulence indicators’ and the Telford strain from Australia.

Remarkably, not all *Map* infected sheep develop clinical disease and the intensity of granulomatous lesions in the intestines (Prez et al. 1996) varies among infected hosts. Based on gross pathology, Ziehl-Neelsen (ZN) staining and histopathology, three pathological forms of *Map* infection are reported viz. asymptomatic, paucibacillary and multibacillary (Smeed et al. 2007). While in the first (most frequent) form an infected host presents no clinical signs of PTb or histological lesions, in the latter two forms, clinical signs and consistent post-mortem lesions of the infection are evident. Paucibacillary and multibacillary forms vary in terms of the number of the acid fast bacilli detected by ZN staining, with a relatively higher number of *Map* pathogens detected in multibacillary lesions.

Differences in disease outcome and infection progression and variation in resultant histological lesions could be attributed to strain differences of the causative pathogen and its associated characteristics such as virulence, immunogenicity and transmissibility. Additional factors associated with the host (genetics, husbandry, infectious dose and environment) could also be significant contributors to disease outcome (Engering et al. 2013). However, in the present study we will not try to account for differences due to host or environmental factors, but will instead focus on genomic characterisation of Type S1 *Map* isolates. We describe the apparent relationship between genotype and some epidemiological factors including region of farm location and some ‘virulence indicators’ that were used to characterize severity of natural *Map* infection under pastoral conditions in adult ewes in our study. These ‘virulence indicators’ referred to three variables viz- histological lesions of *Map* infection, serum ELISA status of the host and provisional diagnosis made

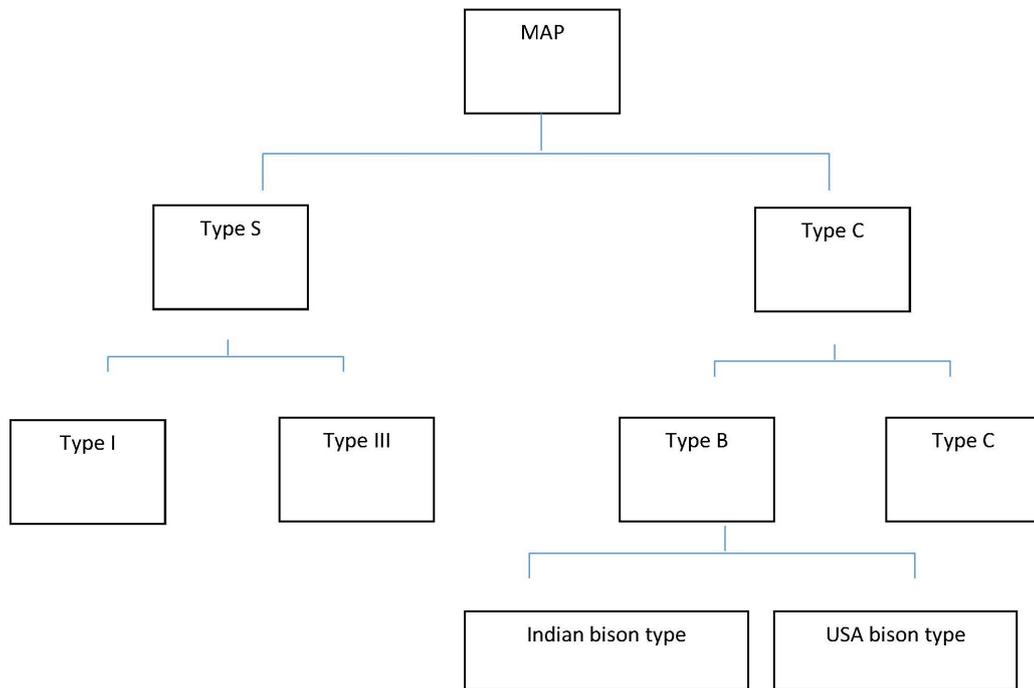
by veterinarians on necropsy examination of *Map* infected ewes.

Infection with *Map* is highly endemic on all major New Zealand livestock farms including sheep. A previous studies has reported 76% of all sheep flocks were infected (Verdugo et al. 2014a). Improved characterization of the *Map* pathogen and a deeper understanding of its epidemiological and molecular features will aid in answering questions about the sources of infection, host-specificity, transmission pathways and geographic distribution and possible differences in virulence of various strains. This understanding may in turn drive the development of improved diagnostic techniques and disease control tools such as vaccines (Grad & Lipcitch 2014).

A previous study characterising *Map* types in New Zealand (Verdugo et al. 2014b) was carried out using traditional methods of genotyping such as the determination of the number of repeats carried at different variable number tandem repeat (VNTR) and short sequence repeat (SSR) loci which are based on repetitive nucleotide sequence differences in the genome of different isolates. The study revealed that the diversity of Type S in New Zealand ovine flocks is much lower than that of *Map* types detected in other ruminants. In general, the robustness of these genotypic methods is influenced by the unstable nature of these repeat regions as markers for *Map* genotyping (Ahlstrom et al. 2015). In contrast, the lack of genetic variability in these repeat regions in Type S isolates from New Zealand (Collins et al. 2011, Verdugo et al. 2014b) limits their use as tools for understanding the true scale of genomic diversity.

Overseas studies have shown that whole genome sequencing (WGS) is superior to VNTR and SSR typing for analysis of *Map* subtypes (Ahlstrom et al. 2015). WGS of bacterial pathogens is increasingly accepted as a routine practice in many labs around the world. This is driven by the fact that the entire WGS process can be completed with relatively low cost and time compared to what was required for this process in the past. As a result, it is now possible to determine the ultimate scale of genotypic diversity and quantify relatedness of infectious bacterial pathogens with relative ease. For a slowly evolving and monomorphic organism such as *Map* (Bryant et al. 2016), this provides an unprecedented opportunity to study within-species diversity of pathogens and understand related sub-characteristics such as virulence and transmissibility.

There are not yet many New Zealand Type S1 *Map* isolates characterised at the WGS level. Therefore, in the present study genotypes of a group of New Zealand Type S1



**Figure 5.1:** Nomenclature for a *Map* phylogenetic analysis based on whole genome sequencing data. C = cattle, S = sheep and B = bison. Adapted from Fawzy et al. (2018).

isolates and the Type S1 Telford 9.2, a closely related and well characterised strain from Australia were compared using Telford1 as a reference genome. Telford1 is a Telford 9.2 strain that was completely sequenced using the PacBio methodology and made publicly available recently (Brauning et al. 2019). The objectives of the this study were: (a) to characterize genomic diversity of Type S1 *Map* isolates in New Zealand based on single nucleotide variant (SNV) analysis; (b) to investigate changes in the genome sequence (SNV) of Telford 9.2 after animal passage; (c) to describe the relationship between *Map* genotype and epidemiological factors (host, farm localisation) associated with the *Map* isolates and (d) to investigate the suspected association of individual genotypes and variables which characterize the severity of natural *Map* infection in host of origin.

## 5.2 Materials and methods

### 5.2.1 *Map* isolates

*Map* isolates selected for WGS in the present study (Table 5.1) were collected from multiple sources as described below. These isolates were all characterised by the VNTR and SSR assay described in Subharat et al. (2012). The VNTR and SSR profile of all isolates matched that of the most common Type S in New Zealand.

#### New Zealand isolates

##### *New Zealand field isolates from a 2012-2014 survey*

*Map* isolates were obtained by culture of lymph nodes and faecal samples collected between August 2012 and June 2014 from 52 ewes, the majority of which were Merinos (n = 35/52) from 17 breeding farms in different regions in New Zealand (Gautam et al. 2018a). These ewes had poor body condition and were suspected to have been affected by clinical PTb based on farmers' diagnosis and therefore were presented for necropsy examination. After histological examination of lesions in the intestinal lymph nodes at New Zealand Veterinary Pathology Limited (Palmerston North, New Zealand), these ewes were grouped into different categories by histopathological scores for intestinal lesions of *Map* infection summarized in Table 5.2. Additional information about other variables associated with these isolates are shown in Table 5.3.

##### *Historical isolates from New Zealand*

This category comprised six *Map* isolates from multiple historical sources: faecal samples originally collected in 1989 from two Merino sheep in Wairarapa and four additional New Zealand sheep isolates (breed information unavailable) for which DNA samples were stored in the DNA archive at the AgResearch, Palmerston North facilities. Since most of the other New Zealand isolates were collected between 2012 and 2014, these historical isolates were included to provide apparent estimates of change in *Map* genotype that could be expected over two decades.

### Australian isolates

#### *Isolates recovered from animal trial (one inoculation strain challenged)*

These strains were isolated at AgResearch Ltd (Palmerston North) from a selection of faecal cultures of nine out of the 56 Merino lambs that were challenged orally with multiple dosages of the Telford 9.2 inoculum (described below), starting at the age of four months (Dukkipati et al. 2016). Telford 9.2 is an *IS1311* Type S1 *IS900* restricted fragment length polymorphism (RFLP) strain from Australia and has been well-characterised both genetically and biochemically and has been used as a challenge strain. These nine lambs were necropsied 63 weeks after their first oral challenge. In all lambs, histopathological lesions associated with *Map* infection were present in the ileo-caecal valve, mesenteric lymph node and liver.

#### *Telford 9.2 inoculum*

Genomic sequences from DNA from the seed stock of Telford 9.2, grown separately in liquid culture medium (M7H9C) and supplemented 7H11 solid medium were obtained.

Both the above group of Australian isolates were Telford 9.2 strains and DNA of these isolates was sequenced at New Zealand Genomics Limited (NZGL), Palmerston North, New Zealand. Given *Map* is a slow evolving organism (Bryant et al. 2016) that offers little genetic variability and scope for genetic recombination under natural conditions (Turenne et al. 2008), the amount of genetic variation between this group of isolates was expected to be minimal. Their inclusion in the present study served as ‘control’ isolates for SNV analyses.

### Reference genome

*Map* Type S strains are distinct from available C strains such as K-10 (National Center for Biotechnology Information (NCBI) reference sequence: NC\_002944.2) by over 3,000 single nucleotide polymorphism (SNPs). With such a large number of differences it is too difficult to accurately identify differences in the more closely related Type S1 isolates being compared for genomic analysis in the present study. A completely sequenced Telford 9.2 genome was therefore prepared for this purpose (Brauning et al. 2019) and its closed genome (Telford1, NCBI reference sequence: CP033688) used as the reference genome

against which all other genome sequences analysed in the present study were mapped for SNV analyses. This work is included as an appendix to the thesis (Appendix B).

### 5.2.2 Other genomes

These genomes for overseas *Map* isolates were available from the Wellcome Sanger Institute (Hinxton, UK) as a result of the study conducted by Bryant et al. (2016). A subset of these genomes including eight Type S [five Type S1 (MAPMRI002-5, 103) and three Type S3 (MAPMRI51, 86, 94)] from four European countries (*viz* Spain, England, Scotland and Czech Republic) were included in our analyses to illustrate the relationship of the New Zealand and Australian Type S1 isolates to their closest known relatives.

#### Reculture and DNA extraction

All New Zealand and Australian *Map* isolates were recultured in a standard 7H9 OADC G T medium which was supplemented with an antibiotic cocktail, Mycobactin, and egg yolk as described by (Whittington 2010). In order to obtain samples that were free of egg yolk, these cultures were then grown on a 7H11 OADC G T antibiotic Mycobactin solid medium. Solid media cultures were heat killed for one hour at 80°C then the bacterial cells were harvested (1/2 plate of bacterial lawn) in 250 µL of 10mM Tris 1mM EDTA (TE) and stored at -80°C.

Genomic DNA was prepared from frozen cells that were thawed and dispersed by aspirating the suspension up and down 10 times through a 26-gauge needle into a 1 ml syringe. The distributed bacterial cells were lysed by adding 20 µL of 10 mg/ml hen egg white lysozyme and incubating at 37°C for two hours. Proteins were solubilised and digested by adding 70 µL of 10% w/v SDS and 12 µL of 10 mg/ml proteinase K and incubating at 65°C for 40 minutes. Bacterial DNA was specifically separated from the other cellular components with a high salt hexadecyl trimethyl ammonium bromide (CTAB) extraction by adding 100 µL of 5 M NaCl, mixing and then adding 80 µL of pre-warmed (65°C) 10% w/v CTAB, 0.7 M NaCl, and then further incubating at 65°C for 20 minutes. DNA was then extracted with 700 µL of chloroform/isoamyl alcohol (24:1) and then centrifuged for five minutes at 20,817 x g in an Eppendorf centrifuge. The top phase was carefully transferred to a new tube and DNA was precipitated by adding 500 µL of isopropanol, mixing

gently and incubating at room temperature overnight. Precipitated DNA was pelleted by centrifugation in an Eppendorf microcentrifuge for 15 minutes at 20,817 x g. DNA was washed 1X with 750  $\mu\text{L}$  of ice cold 70% ethanol and centrifuged at 4°C for 15 minutes at 20,817 x g. DNA pellets were air dried and resuspended in 100  $\mu\text{L}$  of 10 mM Tris pH 8.0. Extracted DNA was quantified with Qubit dsDNA kit (Life Technologies) fluorometry, and was analysed by Nanodrop 1000 spectrophotometry and by gel electrophoresis through 2.5% agarose in Tris borate EDTA (89 mM Tris, 89 mM boric acid, 2mM EDTA) buffer which were then stained with 0.01 mg/ml ethidium bromide in order to assess its purity. Mycobacterial DNA prepared in this way contained contaminating RNA and protein, but collaborators at University of Calgary (UoC) indicated that these contaminants were not likely to interfere with the sequencing. Due to NZGL's concern regarding the accurate determination of DNA concentration, which they feel was required for the Nextera XT library preparation method (Illumina Inc.), DNA that was sequenced at NZGL was treated with RNase for 1 hour following digestion with lysozyme and was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) prior to extraction with chloroform isoamyl alcohol (24:1).

### 5.2.3 *IS900* RFLP typing

A subset of nine New Zealand isolates were typed using the *IS900* RFLP technique. *IS900* RFLP status of the Australian isolates had been predetermined. *IS900* RFLP analysis was conducted by Petra Möbius and colleagues at the Friedrich-Loeffler-Institut (FLI) in Jena, Germany. Genomic DNA (2  $\mu\text{g}$ ) was digested separately with BstEII (Roche Diagnostics) and PstI (New England Biolabs). The DNA fingerprints obtained by *IS900* RFLP were scanned and analysed by using Gel Compare software (version 4.0.11; Applied Maths, Kortrijk, Belgium). All patterns were confirmed by retesting. *IS900* RFLP patterns were analysed visually and after BstEII digestion based on results of Collins et al. (1990) and Möbius et al. (2009). PstI-*IS900* RFLP patterns P1, P13, P22 and P23 were designated according to Whipple et al. (1990), Möbius et al. (2009) and Möbius (2018, unpublished results).

## 5.2.4 DNA Library preparation for WGS

DNA libraries of the New Zealand and Australian isolates were prepared and processed either at UoC or NZGL using either the Nextera or the TruSeq library preparation methods (Table 5.1). Nextera is a transposon-based library preparation method designed for obtaining DNA fragments from small quantities of genomes based on enzymatic shearing (Marine et al. 2011). The TruSeq library preparation method mainly differs from the Nextera XT method in that in the former, DNA shearing is mechanical.

Paired end reads were generated by multiplex sequencing of DNA samples using V2 chemistry (250 bp reads, MiSeq reagent kit).

## 5.2.5 Preparation and processing of the reference genome

A closed Type S1 genome was prepared and processed for use as the reference genome. For this, the Telford 9.2 strain was prepared at the Ramaciotti Centre for Genomics, University of Sydney, Australia. Its frozen bacterial stock was inoculated into M7H9C liquid medium for PacBio sequencing, cultured for 3–4 weeks and then cultivated on modified Middlebrook 7H10 solid medium, harvested and stored at -80°C. DNA extraction was completed using the CTAB method (van Soolingen et al. 1991). For the PacBio sequencing, the DNA was also digested with mutanolysin prior to proteinase K digestion, and subjected to extra clean-up and concentration on Ampure PB magnetic beads.

A PacBio library was constructed and sequenced at the Ramaciotti Centre in Sydney, Australia as described in Brauning et al. (2019). Briefly, a read coverage depth of 80X on the PacBio-RSII platform was achieved on a single-molecule real-time (SMRT) cell using P6-C4 chemistry. The PacBio sequence thus obtained was improved with Illumina MiSeq 250-bp paired-end reads generated by sequencing two cultures of the Telford 9.2 innoculum strain grown separately in the liquid culture medium (M7H9C) and the Herrold's egg yolk medium as described above. Further detail about this reference genome is available in Appendix A.

### 5.2.6 Processing raw reads

All raw sequencing reads of the New Zealand and Australian isolates were subjected to quality control using an in-house Perl script that incorporated SolexaQA++ (version 3.1.17, Cox et al. (2010) and FastQC (Andrews 2010) for read quality analysis and visualisation, Bowtie2 for PhiX removal (Langmead & Salzberg 1923) and the 'fastq-mcf' program from the ea-utils suite of tools for adaptor removal (Aronesty 2011).

### 5.2.7 SNV identification

SNVs in the present study were identified using Snippy (version 3.1, Seemann (2015)). Briefly, Snippy is an open-source pipeline that uses the Burrows-Wheelers Aligner (BWA)-MEM aligner for aligning low-divergent sequences against a single reference genome sequence. The output generated by BWA is then post-processed using SAMtools to generate the sequence alignment map (SAM) and converted to binary alignment map (BAM) files. These BAM files are then utilized by FreeBayes, a Bayesian genetic variant caller to identify small polymorphisms including SNVs. SNVs for multiple isolates that are mapped to the same reference are then used to produce an alignment of core SNVs, i.e. a SNV at a genomic position which is present in all the samples when compared to the reference genome.

In the present study, the processed paired reads from each isolate were aligned to the Telford1 (NCBI reference sequence: CP033688) and SNVs were identified and included in further analyses based on default Snippy settings i.e. a minimum read depth of 10 and a >90% consensus at any potential SNV location. Isolates that achieved <95% coverage of the reference genome were excluded from further analysis.

The list of core SNVs was generated by comparing detected SNVs for all isolates using Snippy-core and imported into SplitsTree (version 4.14.4, Huson & Bryant (2006)) to obtain a neighbour-joining (NJ) phylogram. This phylogram was then exported as a Newick tree for use in Interactive tree of life (iTOL, version 4, Letunic & Bork (2016)). Associated metadata for each isolate in the phylogram were added into the phylogenetic tree in iTOL.

Apart from Snippy, the processed paired reads from each isolate were also aligned to

Telford1 in vSNP<sup>1</sup>, an independent Python-based United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS) SNV detection pipeline for *Map*, *Mycobacterium tuberculosis* complex and *Brucella* species. The vSNP pipeline is similar to Snippy since it also uses BWA as its aligner and FreeBayes as its variant caller. Apart from descriptive statistics of paired reads for each isolates, vSNP produces an SNV table (including both core and non-core SNVs) with associated average *Map* quality scores for each genomic position. Genomic positions, in which there were no missing or mixed nucleotide calls and which had an average mapping quality score of >50 seen across all calls if a SNV was present in that position were retained for comparison with the results from Snippy-core.

### 5.2.8 Further analyses

To further investigate the possibility of that there were detectable correlations between *Map* genotypes and phenotypes, SNVs were categorised by gene function. Functional gene categories for the genes in which SNVs were detected were determined by mapping genes in the Telford1 genome against the phylum Actinobacteria with EggNOG 4.5.1<sup>2</sup>.

In order to determine the relationship between the New Zealand and Australian *Map* isolates and their closest known overseas relatives and to root the phylogenetic tree, paired reads of eight additional Type S *Map* isolates (five Type S1 and three Type S3) from four European countries (Spain, England, Scotland and Czech Republic) described in Bryant et al. (2016) were used. This set of reads from the European countries were available through the Wellcome Sanger Institute. They were included in a SNV identification analysis by Snippy-core along with our 69 isolates and a phylogenetic tree comparison between these two sets of isolates is illustrated in the phylogenetic tree using FigTree (version 1.4.4)<sup>3</sup>.

An additional explanatory variable called ‘combined phenotype of *Map* infection’, which had three levels (Table 5.3), was created by combining numerical scores of the three ‘virulence indicator’ variables (Table 5.4) which we assumed were proxy measures of PTb severity and phenotypic expression of *Map* infection in individual animals (Table 5.4).

<sup>1</sup><https://github.com/USDA-VS/vSNP>

<sup>2</sup><http://eggnogdb.embl.de>

<sup>3</sup><http://tree.bio.ed.ac.uk/software/figtree/>

By combining these three variables we added the scores to create a composite measure of *Map* infection with levels in which the frequency of isolates was more evenly distributed than when the virulence indicator variables were measured individually.

Finally, a distance matrix comprised of pairwise comparisons of SNV differences among the 52 New Zealand field isolates when they were mapped to the Telford1 genome was obtained by importing the multiple sequence alignment files from Snippy-core into Geneious (version 10.1.2, Kearse et al. (2012)). This distance matrix was imported in Primer 6 & Permanova+ (version 1.0.8)<sup>4</sup> for statistical testing of the association between the explanatory variables listed in Table 5.3 and the genotypic dissimilarity between the isolates based on distance matrix of SNVs. Permanova utilizes multivariate analysis of variance (ANOVA) with permutation and allows for statistical testing of the effect of the study variables on the observed genomic alignment. Bivariate analysis was performed to check the statistical significance of association between the *Map* genotype and each of the four explanatory variables listed in Table 5.3.

## 5.3 Results

The group of *Map* isolates being compared here are from two different countries and yet are very similar genetically by two different conventional typing methods. All were previously determined to have the same VNTR and SSR type, and the eight isolates which were successfully characterised by IS900 RFLP strain typing, were all confirmed to be Type S1. The IS900 RFLP profile number of these isolates are presented in Table 5.5. Only one of these isolates, ags36, which was identified as Type S1 by BstEII restriction endonuclease DNA digestion, had a different PstI digestion band pattern when compared to the rest of the isolates (Figure 5.2).

The final data set for SNV analyses included processed paired Illumina reads of 69 *Map* isolates that had achieved at least 95% coverage when aligned to the Telford1 strain in Snippy. Snippy-core identified a total of 672 SNVs in these 69 isolates. Of these, 481 (71.5%) SNVs were unique to one out of the 69 isolates (singleton SNVs), 85 were found in two isolates, 32 in three isolates, 11 in 4 isolates and 63 in at least five or more isolates.

<sup>4</sup><https://www.primer-e.com>

Based on their effect on codons, 393 out of 672 SNVs from Snippy-core were missense or non-synonymous (results in a change in the amino acid sequence including 14 in *IS110* transposase family sequences), 200 synonymous (no change in amino acid sequence), 8 resulted in stop codons (missense mutations that introduced a premature stop codon thereby stopping protein translation) and three were non-coding transcript variants. For SNVs in 68 genomic positions, no such effect on the codon status was determined. When these genomic positions were checked manually in Geneious, 67 of them were in inter-genic regions of the reference genome and one was in a coding gene.

No SNVs were detected in the Telford 9.2 isolates after one passage through a sheep by Snippy-core or vSNP. Based on EggNOG mapping, a total of 501 unique locus tags associated with various functions were identified for SNVs from Snippy-core in 604 different genomic positions. For the rest of the SNVs in 68 different genomic positions no locus tags was identified. The EggNOG mapping functional categories of locus tags and occurrence associated with the 672 SNVs from Snippy-core are listed in Table 5.6.

The phylogram based on the 672 SNVs as determined by Snippy-core and potential associations with other variables associated with the *Map* genomes (Table 5.3) characterised for the present study is shown in Figure 5.3. We found no associations between *Map* genotype and the following variables: histopathological score categories for intestinal lesions ( $p = 0.59$ ), serology status of the host ( $p = 0.51$ ), provisional diagnosis by vets after necropsy examination ( $p = 0.54$ ), or the combined phenotype of *Map* infection,  $p = 0.38$ ). The apparent association between *Map* genotype and region of farm location as shown by clustering of isolates by region (Figure 5.3) was statistically significant in the permanova model with ( $p = 0.04$ ) or without ( $p = 0.001$ ) farm as a random variable. The phylogenetic tree for the 69 isolates from the present study and eight additional isolates from the Bryant et al. (2016) study is shown in Figure 5.3. The New Zealand Type S1 isolates and the Australian Telford 9.2 strain were a genetically distinct group compared to Type S1/S3 isolates from other European countries.

From vSNP analyses, 821 SNVs were identified. Out of these, 44 had average mapping quality score of  $<50$ , and therefore were considered of insufficient quality for further analysis. For the remaining 777 SNVs, a gap (no nucleotide base call) at that genomic position in at least one isolate under study was reported on six occasions (non-core SNV) and mixed base calls (not defined as A, G T or C) in 159 instances respectively. Hence,

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these SNVs were also excluded from further analyses. The remaining 612 SNVs had no low quality, mixed or missing base calls. When these 612 SNVs were compared to the results from Snippy-core, 522 matched in both, while 90 were unique to vSNP. No differences were detected between inoculum and passaged Telford 9.2 by either Snippy-core or vSNP. Compared to 15 SNVs which belonged to *IS110* family transposas from Snippy-core, nine SNVs from vSNP belonged to the same category of the genetic elements.

**Table 5.1:** Sequencing history and other information of the studied isolates sequenced at New Zealand Genetics Limited (NZGL) or the University of Calgary (UoC)

Isolate	Isolate type	Estimated ANC <sup>1</sup>	Venue for WGS	Reference
Nextera library preparton method				
agam12	Telford 9.2 <sup>2</sup>	153	NZGL	Dukkipati et al. (2016)
agam22	Telford 9.2 <sup>2</sup>	185	NZGL	Dukkipati et al. (2016)
agam23	Telford 9.2 <sup>2</sup>	120	NZGL	Dukkipati et al. (2016)
agam28	Telford 9.2 <sup>2</sup>	147	NZGL	Dukkipati et al. (2016)
agam38	Telford 9.2 <sup>2</sup>	122	NZGL	Dukkipati et al. (2016)
agam48	Telford 9.2 <sup>2</sup>	142	NZGL	Dukkipati et al. (2016)
agam4873	historical isolate	176	NZGL	Not available
agam5	Telford 9.2 <sup>2</sup>	116	NZGL	Dukkipati et al. (2016)
agam6758	historical isolate	139	NZGL	Not available
agam80	Telford 9.2 <sup>2</sup>	163	NZGL	Dukkipati et al. (2016)
agam9	Telford 9.2 <sup>2</sup>	133	NZGL	Dukkipati et al. (2016)
agamte	Telford 9.2 (seed stock)	139	NZGL	Not available
agamtl	Telford 9.2 (seed stock)	153	NZGL	Not available
ags01	field isolate	32	UoC	Gautam et al. (2018a)
ags02	field isolate	35	UoC	Gautam et al. (2018a)
ags03	field isolate	37	UoC	Gautam et al. (2018a)
ags05	field isolate	48	UoC	Gautam et al. (2018a)
ags08	field isolate	42	UoC	Gautam et al. (2018a)
ags11	field isolate	35	UoC	Gautam et al. (2018a)
ags13	field isolate	49	UoC	Gautam et al. (2018a)
ags14	field isolate	39	UoC	Gautam et al. (2018a)
ags15	field isolate	39	UoC	Gautam et al. (2018a)
ags17	field isolate	37	UoC	Gautam et al. (2018a)
ags18	field isolate	45	UoC	Gautam et al. (2018a)
ags18h	historical isolate	35	UoC	Not available
ags19	field isolate	62	UoC	Gautam et al. (2018a)
ags20	field isolate	38	UoC	Gautam et al. (2018a)
ags21	field isolate	42	UoC	Not available
ags26	field isolate	43	UoC	Gautam et al. (2018a)
ags27	field isolate	61	UoC	Gautam et al. (2018a)
ags29	field isolate	64	UoC	Gautam et al. (2018a)
ags30	field isolate	30	UoC	Gautam et al. (2018a)
ags31	field isolate	44	UoC	Gautam et al. (2018a)
ags32	field isolate	36	UoC	Gautam et al. (2018a)
ags36	field isolate	37	UoC	Gautam et al. (2018a)
ags37	field isolate	39	UoC	Gautam et al. (2018a)
ags41	field isolate	48	UoC	Gautam et al. (2018a)
ags42	field isolate	32	UoC	Gautam et al. (2018a)
ags44	field isolate	32	UoC	Gautam et al. (2018a)
ags45	field isolate	44	UoC	Gautam et al. (2018a)
ags46	field isolate	46	UoC	Gautam et al. (2018a)
ags47	field isolate	44	UoC	Gautam et al. (2018a)
ags48	field isolate	39	UoC	Gautam et al. (2018a)
ags50	field isolate	43	UoC	Gautam et al. (2018a)
ags51	field isolate	45	UoC	Gautam et al. (2018a)
ags53	field isolate	50	UoC	Gautam et al. (2018a)
ags54	field isolate	33	UoC	Gautam et al. (2018a)
ags57	field isolate	55	UoC	Gautam et al. (2018a)
ags58	field isolate	42	UoC	Gautam et al. (2018a)
ags59	field isolate	72	UoC	Gautam et al. (2018a)
ags60	field isolate	35	UoC	Gautam et al. (2018a)
ags61	field isolate	40	UoC	Gautam et al. (2018a)
ags62	field isolate	40	UoC	Gautam et al. (2018a)
ags63	field isolate	46	UoC	Gautam et al. (2018a)
ags64	field isolate	42	UoC	Gautam et al. (2018a)
ags66	field isolate	52	UoC	Gautam et al. (2018a)
TruSeq library preparation method				
ags04	field isolate	88	NZGL	Gautam et al. (2018a)
ags22	field isolate	77	NZGL	Gautam et al. (2018a)
ags23	field isolate	106	NZGL	Gautam et al. (2018a)
ags24	field isolate	83	NZGL	Gautam et al. (2018a)
ags35	field isolate	129	NZGL	Gautam et al. (2018a)
ags38	field isolate	121	NZGL	Gautam et al. (2018a)
ags43	field isolate	148	NZGL	Gautam et al. (2018a)
ags52	field isolate	94	NZGL	Gautam et al. (2018a)
ags55	field isolate	94	NZGL	Gautam et al. (2018a)
ags56	field isolate	106	NZGL	Gautam et al. (2018a)
h21	historical isolate	144	NZGL	Not available
h22	historical isolate	128	NZGL	Not available
h23	historical isolate	115	NZGL	Not available

<sup>1</sup> Average nucleotide coverage calculated as  $\frac{\{\text{Total number of reads (forward + reverse pairs)} \times 250\}}{\div 4, 907, 428}$ .<sup>2</sup> After a single passage in lamb. WGS = Whole genome sequencing. NZGL = New Zealand Genomics Limited. UoC = University of Calgary.

**Table 5.2:** Summary of intestinal lesions of *Map* infection developed by Prez et al. (1996)

Lesion category	Characteristics of intestinal lesions	Presence of clinical \sub-clinical PTb
1	small focal granulomata of epithelioid cells limited to the Peyer's patches; no macroscopical lesions; no <i>Map</i> detected in Peyer's patches or intestinal mucosa	absent
2	more severe lesions of type 1 in the Peyer's patches, granulomata of epithelioid cells extended to muscosa associated with Peyer's patches; no macroscopical lesions; no <i>Map</i> detected in Peyer's patches or intestinal mucosa	absent
3a	multifocal lesions evocative of early development of type 3b lesions, <i>Map</i> detected in Peyer's patches and the intestinal mucosa	present
3b	diffuse granulomatous enteritis, most advanced lesion types, typical macroscopic lesions visible at necropsy, <i>Map</i> detected in Peyer's patches and the intestinal mucosa; multibacillary	present
3c	diffuse granulomatous enteritis, most advanced lesion types, typical macroscopic lesions visible at necropsy, <i>Map</i> not detected in Peyer's patches, but the intestinal mucosa; paucibacillary	present

**Table 5.3:** Explanatory variables for selected New Zealand *Map* isolates under study

Explanatory variables	Number <i>Map</i> isolates
Region of farm location	
Canterbury	18
Manawatu	9
Marlborough	13
Otago	12
Serology status of ewes based on serum ELISA <sup>1</sup>	
negative (s/p <50)	16
equivocal (s/p >50 and <55)	7
positive (s/p >55)	29
Histopathological score of intestinal lesions	
1	8
2	2
3a	10
3b	24
3c	8
Provisional diagnosis by vets after necropsy of ewes	
OJD negative	8
OJD positive	44
Combined phenotype of <i>Map</i> infection <sup>2</sup>	
0	9
2	18
3	27

<sup>1</sup>*Map* antibody ELISA kit (paratuberculosis screening antibody test<sup>TM</sup>, IDEXX Laboratories. <sup>2</sup>This category was created by adding the numerical scores of the three 'virulence indicators' in the table (serology status of the ewes, histopathological score of intestinal lesions and provisional diagnosis by vets after necropsy of ewes. OJD = ovine Johne's disease. s/p = sample-to-positive)

**Table 5.4:** Categories of explanatory variables and scores for phenotypic expression of PTb severity due to *Map* infection for selected New Zealand isolates under study

Explanatory variables	Given score
Histopathological score of intestinal lesions	
1	0
2	0
3a	1
3b	1
3c	1
Serology status of ewes based on serum ELISA <sup>1</sup>	
negative (s/p <50)	0
equivocal (s/p >50 and <55)	0
positive (s/p >55)	1
Provisional diagnosis by vets after necropsy of ewes	
OJD negative	0
OJD positive	1

<sup>1</sup>*Map* antibody ELISA kit (paratuberculosis screening antibody test<sup>TM</sup>, IDEXX Laboratories. OJD = ovine Johne's disease. s/p = sample-to-positive )

**Table 5.5:** Strain types by *IS900* RFLP of selected *Map* isolates

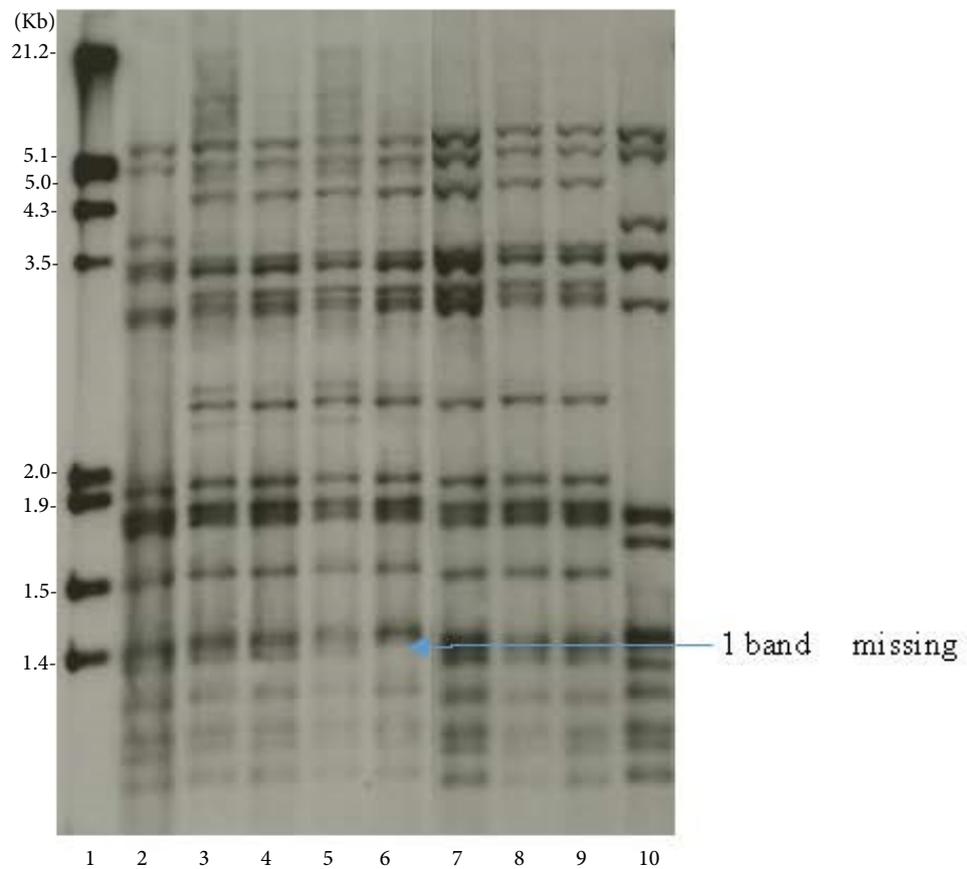
Isolate	VNTR/SSR profile	<i>IS900</i> RFLP	PstI profile
agam6758	41331118 / 3	S1 (BstEII)	P22 (PstI)
agam4873	41331118 / 3	no result	no result
ags2	41331118 / 3	S1 (BstEII)	P22 (PstI)
ags8	41331118 / 3	S1 (BstEII)	P22 (PstI)
ags15	41331118 / 3	S1 (BstEII)	P22 (PstI)
ags17	41331118 / 3	S1 (BstEII)	P22 (PstI)
ags36	4133(-)(-)-18/Nd	S1 (BstEII)	P23 (PstI)
ags57	41331118 / 3	S1 (BstEII)	P22 (PstI)
ags63	41331118 / 3	S1 (BstEII)	mix (PstI)

RFLP = restricted fragment length polymorphism, Nd = Not detected, - missing in the VNTR loci under investigation

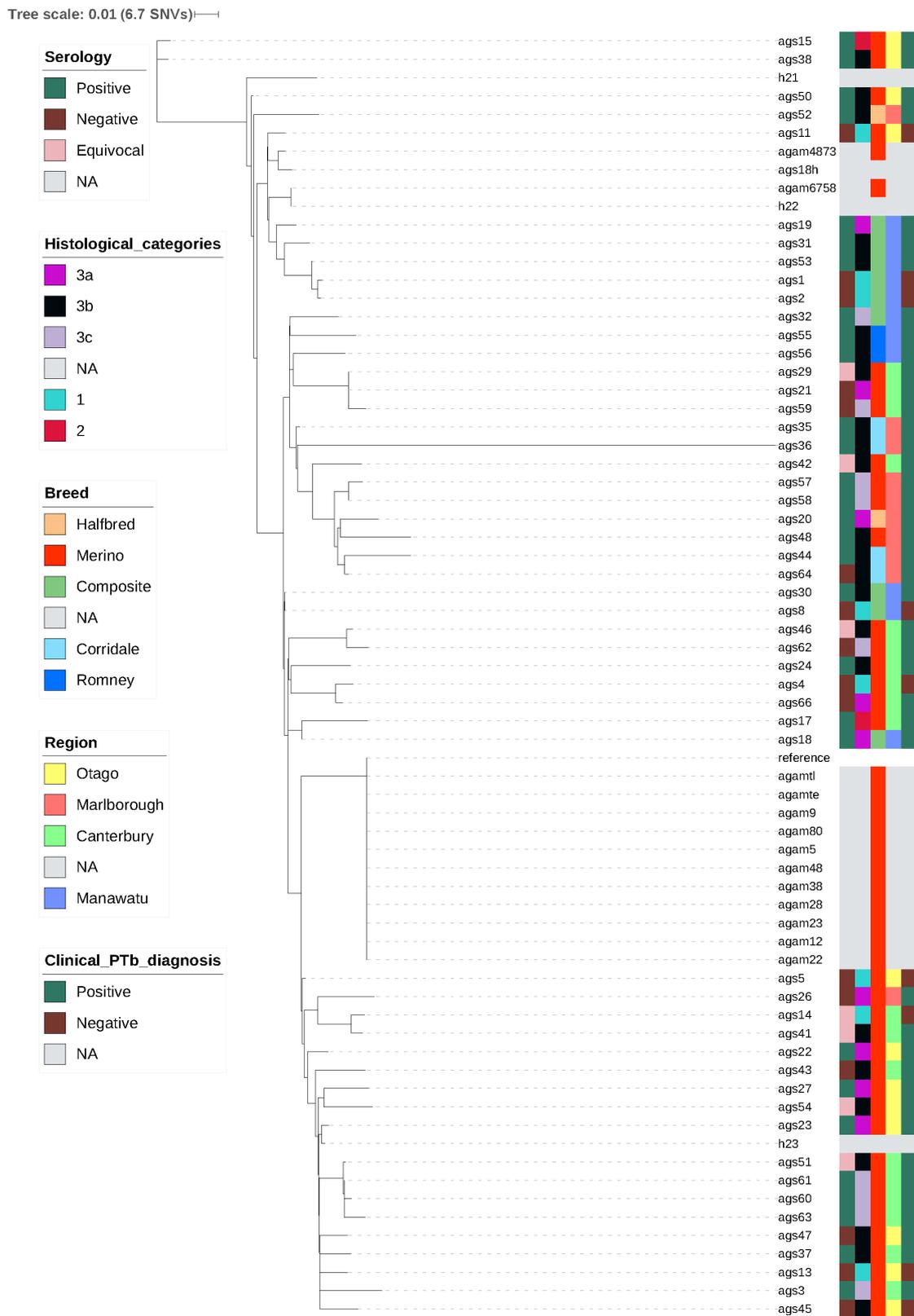
**Table 5.6:** The EggNOG<sup>1</sup> mapping functional categories of locus tags and occurrence associated with the 672 single nucleotide variations (SNVs) from Snippy-core

EggNOG functional categories and functions	EggNOG code	Occurrence of SNVs per EggNOG category
Cellular processes and signalling		
cell cycle control, cell division, chromosome partitioning	D	2
cell motility	N	1
cell wall/membrane/envelope biogenesis	M	13
cytoskeleton	Z	1
defense mechanisms	V	4
post-translational modification, protein turnover, and chaperones	O	14
signal transduction mechanisms	T	14
Information storage and processing		
replication, recombination and repair	L	35
transcription	K	33
translation, ribosomal structure and biogenesis	J	7
Metabolism		
amino acid transport and metabolism	E	41
carbohydrate transport and metabolism	G	20
coenzyme transport and metabolism	H	9
energy production and conversion	C	34
inorganic ion transport and metabolism	P	42
lipid transport and metabolism	I	44
nucleotide transport and metabolism	F	8
secondary metabolites biosynthesis, transport, and catabolism	Q	68
Poorly characterized		
function unknown	S	147
Miscellaneous		
SNVs in intergenic regions (no locus tags) in EggNOG mapping	67	
SNVs in intragenic regions (no locus tags) in EggNOG mapping	32	
SNVs in intragenic regions for which functional category was not specified in EggNOG mapping	32	
SNVs in intra-genic regions for which more than one functional category was identified		4
Total SNVs in Snippy-core		672

<sup>1</sup> <http://eggnogdb.embl.de>. SNV = single nucleotide variant



**Figure 5.2:** IS900 RFLP profiles of various *Map* isolates from ewes after digestion with restriction endonuclease PstI. From left to right: 1 = molecular weight marker (size in kilobytes), 2 = JIII-386 (Type S3 German strain), 3 = ags57, 4 = ags8, 5 = ags17, 6 = ags36, 7 = agam6758, 8 = ags2, 9 = ags15 and 10 = 362 (Type C New Zealand strain). Compared to all other New Zealand strains ags36 had a band missing as indicated by the arrow.



**Figure 5.3:** Whole genome SNV-based phylogenetic tree of Type S1 *Map* isolates from New Zealand and Telford 9.2 strain from Australia. The tree was created using iTOL version 4 (Letunic & Bork 2016) based on SNVs identified through mapping all isoaltes to the Telford1 reference described in the text. NA = Not available



## 5.4 Discussion

In the present study we characterise Type S1 *Map* isolates in New Zealand and Telford 9.2 strain from Australia based on WGS data. A similar number of SNVs were detected by two different methods with a total of 672 core SNVs in 69 genomes by Snippy-core compared to 612 by vSNP. We did not detect evidence of an association between *Map* genotypes and the following factors that were examined in this study: serum ELISA status of the host, histopathological categories of *Map* intestinal lesions due to *Map* infection and provisional diagnosis made by the vets after necropsy. There was an association between the *Map* genotype and region of farm location. The New Zealand and Australian *Map* isolates analysed in the present study *Map* were closely related and therefore analysis for homoplasy that might have indicated selection for virulence was not undertaken. Additional data analyses including gene ontology (GO) enrichment for identification of gene functions in the associated lineages and the Pfam analyses for functional annotation of the genomic data from the Type S1 isolates from New Zealand has been planned and will be presented separately.

SNV-based analyses are particularly useful for pathogens such as *Map*, which exhibit limited genetic diversity (Bryant et al. 2016). For *Map*, SNV-based analyses are superior to other genotyping techniques such as VNTR and SSR that use repetitive DNA elements for the study of phylogenetic relationships (Leo et al. 2016, Ahlstrom et al. 2016). Although WGS does a better job of distinguishing Type S *Map* isolates than conventional genotyping methods, even when characterised with the ultimate resolution provided by WGS, *Map* is slow to incorporate new changes into its genome. This may be because of the limited number of imported *Map* strains into New Zealand because of its isolated position and short history of PTb.

In the present study, we sequenced genomes of Type S *Map* isolates from several sources. The majority of field *Map* isolates in the present study came from a previous New Zealand study that estimated ewe mortalities due to clinical PTb (ovine Johnes disease, OJD) on affected farms (Gautam et al. 2018a). Associated metadata such as farm location, histopathological scores of intestinal lesions and serology ELISA status were available only for this subset of samples. Nevertheless the inclusion of historical New Zealand isolates and a subset of Telford 9.2 isolates from the experimental trial (Dukkipati et al.

2016) for which associated metadata were not available allowed us to compare SNVs from a larger set of *Map* genomes. This approach to include isolates collected at different times and locations for SNV analysis might have also helped to reduce the sampling and selection bias in our study. Furthermore, all isolates from (Dukkipati et al. 2016) were strains resulting from one passage of the Telford *Map* Telford 9.2 through sheep and therefore little, if any SNV variation in this group of Australian isolates was expected based on the Bayesian estimates of *Map* mutation rate (Bryant et al. 2016). This expectation was met as our analyses showed no SNV variation among the Australian isolates regardless of the culture media (liquid or solid), a single passage through different hosts and variation in the sequencing platform (PacBio versus Illumina). Therefore all these Telford isolates were retained in the final analyses as ‘internal controls’.

The investigation of the history of Type S1 *Map* isolate evolution was not the primary objective of the present study. Nevertheless eight additional *Map* isolates from a global comparison of *Map* types by WGS (Bryant et al. 2016) were included along with the 69 isolates in the present study for SNV analyses for a phylogenetic comparison of New Zealand-Australian and overseas Type S1/S3 isolates (Figure 5.4). The figure illustrates that Type S1 isolates from New Zealand and Australia are closely related to one another and more distinct when compared to European Type S1 or S3 isolates. These results help to validate our choice of Telford1 as the appropriate reference genome for SNV analysis. A study of microevolution of the Type S1 *Map* in New Zealand sheep could be an interesting field for future research.

We observed that SNVs were apparently scattered in different locations within and between genomes of different isolates. The SNVs did not show apparent clustering with the three factors (‘virulence indicators’, Table 5.4), which were the phenotypic measures of *Map* infection in our study. We observed that SNVs in 67 genomic positions by Snippy-core analyses were located in intergenic regions of the reference genome. Such SNVs might be associated with disease phenotypes, but it is more challenging to understand their association with disease phenotypes compared to SNVs within a gene of known function (Chen & Tian 2016).

Based on SNV analysis, there was also a lack of genetic signal for selection of any particular gene or a functional category of genes, indicating that the SNVs were likely to have resulted from random genetic drift, a mechanism of evolution in which sampling

error leads to change in allele frequencies of a population, rather than as a result of gene flow. Approximately 71% of the SNVs in the present study were singleton SNVs. By comparison to the SNVs shared by more than one isolate, singleton SNVs are more likely to be recently acquired changes without the same selection pressure as the SNVs shared by more than one isolate for slightly deleterious mutations to be removed from the population. Some of these SNVs may have occurred during the laboratory culture of the organisms. The statistical significance of association between *Map* genotypes and region of farm location observed in this study is most likely caused by animal movement for trade among farms within regions which helps to establish *Map* with a more similar genotype within individual regions (Marquetoux et al. 2016).

A study of Type C *Map* isolates from dairy cattle in Germany did not find associations between *Map* genotypes and presence or absence of PTb typical macroscopic lesions in the host (Möbius et al. 2017). The German study was conducted in Type C isolates that were genotyped by IS900 RFLP (with two digestion enzymes) and VNTR analysis. No difference in the virulence of *Map* genotypes could be detected. At the level of difference in the *Map* isolates that we had in the present study and those that were presented in the Möbius et al. (2017) study, results in terms of *Map* genotype and disease progression were similar and both these studies lent support to the hypothesis that factors other than the genotype of the *Map* pathogen might have a larger influence on the host-pathogen interaction and finally on the severity of disease progression. These factors may include among others host genotype, the initial infection doses or the duration of host contact with *Map*-infected environments.

It is noted that the small sample size used in this study would not allow us to completely discard the possibility of virulence difference within Type S1 and therefore the observed phenotypic difference in PTb severity among *Map* infected sheep. Furthermore, a longitudinal study would allow collection of multiple samples from same animal and the long term follow up would give more assurance that an animal would or would not develop PTb compared to the cross sectional approach taken in this study. Investigation of characteristics of *Map* other than SNV analyses (for example the methylation of specific areas in the genome of individual isolates) might be useful to investigate the observed phenotypic difference in PTb severity among *Map* infected sheep.

In the present study the majority of intestinal lesions belonged to subtypes of type 3

(Prez et al. 1996), which represented a more severe (clinical or sub-clinical) form of *Map* infection. This lack of variability among the histopathological lesions was to be expected because the ewes that were presented for necropsy examination were in poor body condition and the farmers suspected they were affected with clinical PTb. Hence they were most likely to be categorised as histopathological type 3s. We detected some ewes with type 1 lesions, the mildest of all lesion categories, but Type S *Map* culture is difficult and less efficient when there are low numbers of viable *Map* cells present. Thus we were probably not able to isolate *Map* from all lesions belonging to this category. In addition, some of the type 1 lesions are likely to have resulted from infections with other strains of *Map* that were not able to grow in the culture media used. Overall this resulted in a lower number of isolates from type 1 or type 2 intestinal lesions. It is acknowledged that sampling ewes in poor body condition for collection of faecal and lymph node samples in the present study may have resulted in *Map* isolates from a biased subset of hosts that was less likely to include low shedding animals and supershedders without clinical signs of the disease. As a consequence it is possible that a lesser genotypic variation was captured than originally hypothesized. However, it is notable that genotypes from historical *Map* isolates in New Zealand and the Telford 9.2 strain after a single passage in lambs were not different to the field *Map* isolates collected for the purpose of this study, thereby providing additional credibility to our current results. If a larger genotypic variation in New Zealand Type S *Map* isolates exist, it would be better captured by isolation and comparison of *Map* isolates from a larger number of hosts varying in terms of host *Map* status [for example infected versus infectious (shedding but not clinically affected) versus diseased; reviewed by Whittington et al. (2017)].

The identification of SNVs in the present study was based on *in silico* analysis only and we did not perform PCR to confirm them since it was not within the scope of the study to propose any genetic markers. Despite a relatively homogenous genotypic structure of isolates the number of SNVs in one isolate, ags36, was much higher than the rest of the isolates. IS900 RFLP typing confirmed that the isolate was Type S1, but had a slightly different PstI profile (Figure 5.2). Identification of SNV markers in Type S1 *Map* genomes could be undertaken as a separate research study in the future.

The *de novo* (reference free) assembly of paired Illumina reads proved to be difficult for these *Map* genomes warranting the need for reference based mapping for genomic com-

parisons. When we tried *de novo* assembly of one of the field isolates using SPAdes (Bankevich et al. 2012), the quality of resulting assembly was deemed unsatisfactory (results not shown). Because it also required assembly, results of using a reference free method called kSNP (Gardner et al. 2015) for SNV identification were also deemed unsatisfactory. *Map* genomes have a relatively high GC content, approximately 70%, and are characterised by the presence of repeat regions called the IS elements. These elements can be several kilobases long and occur in multiple locations thereby posing a significant challenge in precise assembly of contigs in the absence of a proper reference. When this study began, the closest available reference was K-10 (NCBI reference sequence: NC\_002944.2), a closed genome from a Type C isolate. This K-10 isolate differed from the New Zealand and Australian Type S1 *Map* by over 3,000 SNVs (Bryant et al. 2016) and was not ideal for this work. The Telford1 reference generated to facilitate these comparisons differed from all isolates by 0–164 (median 40) SNVs and was superior for these analyses.

## 5.5 Conclusions

Type S1 *Map* isolates from New Zealand and the Telford 9.2 strain from Australia are closely related genotypes (range of SNVs 0–164 when compared to the same reference) and both the New Zealand and Australian isolates are distinct from Type S1 and S3 isolates from other countries in Europe. The *Map* genotypes and region of farm location were significantly associated indicating a regional localization of genotypes most likely dictated by a close network of animal movements within regions. No apparent associations were observed between *Map* genotypes and phenotypic expression of *Map* infection based on histopathological scores or humoral immune response (ELISA) of the host. This suggested that there were little or no virulence differences among *Map* isolates of this study. Hence, *Map* virulence may not be an important determinant of disease outcome in infected sheep.

## 5.6 Acknowledgements

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## General discussion

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The present research is the sixth and so far the most recent PhD project completed at the School of Veterinary Science (previously known as the Institute of Veterinary, Animal and Biomedical Sciences) at Massey University that investigated aspects of paratuberculosis (PTb) epidemiology in at least one major livestock species (dairy and beef cattle, deer and sheep) in New Zealand. The focus of this project was on commercial sheep flocks and the main objective was to understand the epidemiology of ovine PTb in New Zealand in a more comprehensive manner than reported previously.

In order to achieve this main objective, several secondary objectives were specified and investigated by individual research studies involving (a) review of current clinical PTb control programs in New Zealand; (b) economic analysis of vaccination as a control method of clinical PTb in affected flocks; (c) diagnosis and determination of *Map* shedding and exposure at individual animal levels in commercial flocks using qPCR and serum ELISA respectively and (d) genomic characterization of Type S1 *Map* genomes in New Zealand based on the analysis of whole genome sequence (WGS) data. This thesis as a combined document fills several gaps in existing knowledge about ovine PTb in New Zealand. The results presented here will be of interest to individuals and organisations that are involved in developing new or existing ovine PTb control activities.

These above specific objectives were identified based on knowledge gaps existing in the current understanding of ovine paratuberculosis in New Zealand context and results presented in this thesis will help to further the knowledge regarding the control of PTb in New Zealand. For instance, the comprehensive review of the current PTb control measures in New Zealand helped to understand and assess how various livestock industries have prioritized PTb control and how overall PTb control measures in this country com-

pared to those practiced in other developed nations in the world. Similar estimates of OJD attributable mortality in adult ewes based on longitudinal data of ewe mortality and benefit cost of OJD vaccination estimates in clinically affected flocks provided useful parameters for future modelling studies focused on PTb control in New Zealand sheep flocks. Estimates of intraflock *Map* shedding and prevalence of *Map* antibody positivity in non-clinical ewes provided insights into expected burden of *Map* infection at animal level in non-clinical flocks and together with the ewe mortality estimates, will also be useful parameters in modelling studies for PTb control. The closed Telford1 genome processed and used as a Type S reference genome in the present research will be a useful Type S reference for future molecular studies in New Zealand and overseas. Finally, using the ultimate genomic resolution provided by WGS– at the nucleotide level– we characterised Type S1 *Map* isolates and this helped to gain a deeper insight about the molecular epidemiology of *Map* in New Zealand.

## 6.1 Scope of thesis chapters

In chapter two, the focus was to review existing clinical PTb control measures implemented by different livestock industries in New Zealand including the dairy/beef cattle, deer and sheep industries. The brief history, background and general status of PTb in New Zealand in the specified sectors was also described. This is the first and so far, the only peer-reviewed publication focused on PTb control in New Zealand livestock. Data presented in this review were mainly based on publications in peer-reviewed journals, preferably from New Zealand based studies, and overseas studies if the latter was deemed necessary. Specific information about PTb in New Zealand did not always exist in peer-reviewed references, therefore several non-peer-reviewed sources considered as robust by the authors (Gautam et al. 2018b) were also cited.

In chapter three, annual ewe death records from 17 voluntarily participating sheep breeding farms, which were known to be affected with clinical PTb and located in different regions of the country were used to estimate the annual ewe death rate attributable to clinical PTb. These data collected over 30 farm-years were then used as parameters to evaluate the cost-benefit of vaccination against PTb using a stochastic simulation.

In chapter four, we present results of a study in which frozen faecal (n = 878) and serum

samples ( $n = 837$ ) from clinically normal ewes from 45 flocks were tested by real-time qPCR and ELISA respectively in order to determine faecal shedding and serology in commercial flocks in New Zealand. The quantitative relationship between serum ELISA and qPCR was assessed.

Finally in chapter five, we characterised 56 sheep Type S1 *Map* isolates from New Zealand and 13 isolates of Telford strain from Australia based on single nucleotide variant (SNV) analyses using whole genome sequence (WGS) data. The majority of these isolates were cultured from intestinal lymph nodes or faecal samples from ewes, chosen for post-mortem by farmers as they were suspected to have clinical PTb (described in Chapter 3). A closed (completely sequenced) Type S1 genome (Telford1) was generated and used as a reference for comparison of SNVs in these isolates. The association between *Map* genotype and phenotypic expression of PTb defined by different categories: histopathological scores of intestinal lesions, serology status and provisional diagnosis made by a veterinarian was assessed.

While the chapters of this thesis may at first may seem relatively unrelated, and therefore a cohesive synopsis and discussion appeared to be difficult the general reason for the individual research chapters was to fill research gaps that were identified by previous (for example, Verdugo (2013)) or parallel (for example, Marquetoux (2017)) work that led to hypotheses for future research that were specific to New Zealand. The findings from the combined set of data generated in this thesis have helped in understanding and drawing evidence-based inference on several aspects of ovine PTb in New Zealand. It is acknowledged that sampling strategy and study designs undertaken for independent research chapters presented in this thesis had some inherent sampling bias or study limitations associated with them, which have been discussed and acknowledged separately in the respective chapters. Whenever possible, efforts were made to address these issues through the use of more robust methods for data processing and analysis (for example stochastic simulation presented in chapter 3), qualitative assessments of bias that might have existed (chapter 4) or inclusion of samples or raw data from other studies which would enable comparison of results from a wider source of data (chapter 5).

## 6.2 Current status of PTb control in New Zealand

A number of factors regarding PTb in New Zealand are worth noting. Unequivocal estimates of *Map* infection are not available for all species, but the infection is widespread with at least 40% of all major livestock flocks/herds infected (Gautam et al. 2018b). Despite this widespread prevalence of infection, the average annual incidence rate of clinical PTb in infected herds/flocks is less than 0.5% in all species (Gautam et al. 2018b). Nevertheless, as shown in the results of chapter three in this thesis, the annual rate of clinical disease can be much higher, but this occurs in a smaller number of farms. A national PTb control program does not exist and each livestock industry has different priorities about PTb control. The deer industry clearly is the most proactive. Its levy-funded agency Johne's Management Ltd. operates through a network of specifically trained veterinarians who assist farmers in the control of the disease. Control programmes exist for dairy cattle and sheep, but their uptake is far lower compared to other countries. PTb in beef cattle is rare and no control programmes are currently offered to beef farmers.

Most dairy farmers in New Zealand do not see PTb as a major impediment to productivity (Norton 2007). The general perception among sheep farmers is not different. Given that the economic burden of PTb to livestock industries appears to be relatively low, the voluntary approach by New Zealand farmers to reduce the incidence of clinical PTb and decrease the overall prevalence of *Map* infection in their farms based on best management practices is justifiable for all livestock species.

Paratuberculosis in New Zealand has a long history. The first diagnosis was in an imported Jersey cow in 1912, but it was not until 16 years later in 1928, that two cases were identified in local dairy cattle in the Taranaki district (Stephens & Gill 1937). The first control initiative was undertaken in the 1930s by listing PTb as a notifiable disease and this status was maintained until 2000 (de Lisle 2002). However, the lack of good diagnostic tests and unimproved farming practises impaired the success of this early effort (de Lisle 2002).

The most recent national-scale coordinated effort to address the PTb issue was through establishment of the Johne's Disease Research Consortium (JDRC, 2008–2016). Jointly funded by the New Zealand Government and livestock industries, JDRC was a partnership between various research providers and industries. The overall objective of the consor-

tium was to develop practical cost-effective tools for reducing overall prevalence of PTb across different livestock sectors. In order to achieve this objective, four broad research areas including epidemiology, genetics, pathobiology and diagnostics were identified and as an outcome of the joint venture, PTb control guidelines with recommended management tools are now available. JDRC was undoubtedly a key initiative that helped answer a broad range of PTb related questions in a New Zealand context and facilitated development of PTb control plans in the major livestock industries. Nevertheless, despite a substantial JDRC tenure of approximately eight years, an unequivocal estimate of disease prevalence and clinical disease incidence in dairy cattle and sheep in New Zealand are still not available. Such estimates would have helped to provide the means for a targeted reduction of the disease incidence and prevalence.

Additionally, the cost-benefit estimates of participation in a voluntary PTb control and prevention program and overall efficiency of such methods in achieving control over PTb remains to be undertaken for all species. In the United States of America, such programs focused on cattle were reported to be cost-effective (Wolf et al. 2014). The knowledge of expected costs and benefits due to involvement in a disease control program can aid farmers in making informed decisions regarding their participation (Wolf et al. 2014). More importantly, measurable evidence of success of such voluntary disease control programmes will contribute to the sustainability of these programs and a wider acceptability by the farming communities. This could be an area for future PTb research in New Zealand.

The biggest threat arising from PTb in New Zealand would be public health and trade barrier impacts should it be recognised as a food safety hazard. Although a large number of studies (reviewed by Feller et al. (2007), Abubakar et al. (2008)) have reported association between *Map* and Crohn's disease in humans, the causal associations between them remains unproven. Therefore, the zoonotic risk of *Map* based on currently available evidence would only be an assertion rather than a reality. It should be noted, however, that *Map* has been cultured from pasteurized milk, commercial infant formula and beef (reviewed by Kuenstner et al. (2017)) from several countries and from muscle and blood of ewes without clinical PTb in New Zealand (Smith et al. 2011). Apart from the ethical concern, the presence of a potentially infectious pathogen in food for human consumption is not desirable. Even in the absence of a mandatory control program, limiting the

prevalence of infection on-farm based on good farming principles is likely to reduce or obliterate *Map* from the final food product.

A recent example of the impact that PTb can have on trade was seen in 2016, when Japan placed a temporary ban on live cattle imports from Australia after a shipment of cattle from Melbourne tested positive for PTb<sup>1</sup>. New Zealand banned the export of live animals for slaughter in 2003, but allows export for breeding. While New Zealand has never faced a similar PTb trade predicament, trade competitors of New Zealand such as Australia and the United States have adapted stricter PTb management programmes (Geraghty et al. 2014) than New Zealand, providing them a head start should a need for a national PTb control program arise in the future.

### 6.3 Economic impacts of ovine paratuberculosis

The reported estimates of economic losses due to PTb in various countries are different. The variation in these estimates is partially attributable to difference in prevalence of disease, production system in a herd, herd size, management practices and underlying assumptions of calculations (Hasonova & Pavlik 2006). For instance, in the USA the average annual cost of PTb was US \$200–250 million or US \$22–27 per cow (Ott et al. 1999). By comparison, in the UK the average annual cost of PTb/cow was GB £27 (Gunn et al. 2004) and in Australia it was A \$45 per cow per year (Shephard et al. 2016). In New Zealand the annual loss to the dairy industry was estimated to be at least NZ \$3.8 million, but could be as high as NZ \$18.9 million (Brett 1998). However, the validity of this New Zealand-based study was limited by factors such as the lack of robust estimates of disease prevalence, limited performance of the diagnostic tests used to detect infection and exclusion of the sub-clinical effects of PTb.

Unlike the situation in dairy cattle, the economic cost of PTb in other livestock species including sheep has only been reported in a limited number of studies. Apart from the economic analyses of the cost of PTb in the dairy, beef, sheep and deer industries by Brett (1998), no other study has estimated the cost of PTb in New Zealand. Compared to New Zealand, a larger number of ovine PTb studies have been conducted in its neighbouring

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<sup>1</sup>[https://www.nzherald.co.nz/nz/news/article.cfm?c\\_id=1&objectid=11463095](https://www.nzherald.co.nz/nz/news/article.cfm?c_id=1&objectid=11463095)

country, Australia. Given the similarities between New Zealand's predominantly pastoral livestock system and extensive grazing system for livestock practiced in a large part of Australia (Henry et al. 2012), plus a comparable animal health status in these two countries, ovine PTb research studies conducted in Australia might be the next best references to apply to a general understanding of the economic impacts of ovine PTb in the absence of New Zealand-specific data.

Our estimates of OJD-attributable mortality presented in chapter three of this thesis was primarily derived from ewe mortality rates and causes of mortality in voluntarily participating farms that were affected by clinical PTb. These rates were based on annual farmer-enumerated monitoring data of a two-year observational study. Individual animal level demographical and production data are usually not maintained by sheep farmers, but by some farmers maintained at the flock level. In a 2012 sheep-farmer survey, only 28% farmers reported using ear-tags in their sheep and only 5.9% used electronic tags (Corner-Thomas et al. 2015). The lack of monitoring flock performance is probably due to the general inconvenience in observing, recording and maintaining individual animal records. In this study, twenty farmers agreed to participate initially, but mortality records from only 17 farms were finally available to be used for analysis. Mortality data from three farms were deemed incomplete. While collection of data from 85% (17/20) of the farms initially enrolled in our study was highly acceptable, it is noteworthy that all of the 20 farms were clinically affected by PTb. In order to motivate farmers to maintain accurate data, it might be necessary to demonstrate additional production incentives which cover, or at least partially cover, the time and resource required for the job of accurate data keeping. In particular the use of electronic ear-tags greatly enhance the ability to collect individual-animal data (Corner-Thomas et al. 2015) and must be encouraged. Additionally, a predefined case definition for reasons of death and removals and consistent use across farms will ensure more accurate and consistently recorded reasons of death in sheep farms. Stochastic simulation models can aid in making decisions regarding the choice of PTb control methods in a flock, based on formal economic analysis, but accurate information on demographical and production details of animals are prerequisites for credible simulation outcome. Hence proper and accurate data recording on farms is important.

We observed that the OJD-attributable mortality rates varied by farm (IQR 0.3–2.7%).

While most participating flocks had fine-wool (e.g. Merino) breeds ( $n = 13/17$ ), and were therefore a reasonable representation of the OJD-affected fine-wool farms, they were not typical of commercial sheep farms in New Zealand since the fine-wool breeds make up only  $<10\%$  of sheep in New Zealand (Beef and Lamb New Zealand 2017, West et al. 2017). We intended to extrapolate our OJD mortality estimates and benefit-cost analyses of vaccination to the general ovine population and to estimate the national cost of OJD by stochastic simulation modelling. The model was informed by the parameters generated in this study. A few additional assumptions were required about input parameters such as farm gate prices, purchase and replacement cost. The advantage of simulation modelling is that uncertainties of input parameters are reflected in the final outcomes shown as distribution densities. We acknowledge the inference about costs of ovine PTb in New Zealand should be based on a larger and more representative sample of farms across the country. Robust data on mortality due to clinical disease and loss of production due to subclinical PTb collected over several years would ideally be obtained to estimate a more accurate, precise and representative impact of the economic costs of ovine PTb. Nevertheless, our estimates were able to substantiate the relative merit of vaccination relative to the level of clinical PTb in a typical New Zealand flock. It generated the ‘thumb rule’ that, by and large, vaccination is likely to pay-off on fine-wool farms when about 1% of ewes are lost due to PTb every year.

While OJD remains an important production disease for sheep flocks in New Zealand, especially for fine-wool breeders, it is not a disease with high priority for control. Since the need for a national PTb control program has not been warranted for any species (Brett 1998) and the disease is not currently notifiable (de Lisle 2002), it is logical that only a small number of farms where clinical disease is considered an issue would adopt a control method. However, due to the lack of monitoring and likely under-diagnosis of OJD, it is likely that farmers often fail to appreciate the potential impact. The situation is not much different in Australia where estimates of annual OJD mortality in affected sheep flocks are mostly based on records provided by farmer-reported data and vary largely by geographical region (Bush et al. 2006). Since *Map* shedding can occur before an animal tests positive or shows signs of clinical disease (Fecteau et al. 2013), infection will often have already established in the flock before a formal diagnosis is made. In self-replacing flocks, a routine surveillance using a combination of ante-mortem diagnostic methods

such as the PCR and the serum ELISA might be helpful to monitor the flock status. In non self-replacing flocks where replacement ewes need to be purchased from other farms, the history of PTb and the current flock status of the source flocks should be established before animals from such a farm are introduced into the flock.

## 6.4 PTb diagnosis using serum ELISA and qPCR

Serum ELISA, faecal culture and PCR are common ante-mortem diagnostic tools for PTb. While the former targets the detection of immune antibody response to *Map* infection, the latter two detect the presence of *Map* antigen. Diagnosis of PTb in infected animals without clinical signs of disease is marred by limited sensitivity of current diagnostic techniques. Experimental studies have shown that humoral immune response is preceded by cellular immune response (IFN $\gamma$ ) in the early stages of exposure to *Map* (Begg et al. 2010, Dukkipati et al. 2016), and might have a higher sensitivity than the serological tests that measure humoral immune responses (Huda et al. 2004). However, diagnostic tests that measure cellular immune responses are not commonly used as routine diagnostic tools (Eamens et al. 2015). Faecal shedding usually starts much later after *Map* infection (Dukkipati et al. 2016). Both antibody response and faecal shedding can be intermittent in nature (Stewart et al. 2004) particularly in early stages of infection, thereby affecting the sensitivity of serum ELISA and faecal culture. Thus, the utility of any currently available test for the diagnosis of PTb is very limited.

In chapter four of this thesis we estimated the animal-level prevalence of *Map* shedding and serum antibody positivity exposure based on qPCR and ELISA testing of faecal and serum samples respectively in clinically normal ewes in New Zealand pastoral conditions. These ewes were sampled from 45 commercial farms with a pre-determined pooled faecal culture (PFC) status described in Verdugo et al. (2014a). We also observed a higher sensitivity of sampling individual animals by PCR and serum ELISA over sampling a single pool of 20 random animals for faecal culture as a tool for determining flock-level status. The serum and faecal samples were a subset of a larger survey conducted for the purpose of a previous PhD study in 2010 (Verdugo 2013) and were stored at -80°C since then. By using the combination of PCR and ELISA on samples from same individual animals we found a higher prevalence in the flock than was previously estimated. When

examined at the individual animal-level by serum ELISA and faecal qPCR, 44% and 46% of flocks pre-diagnosed as negative by PFC in Verdugo et al. (2014a) were positive by PCR and ELISA, respectively. It is noted that overseas studies have reported a much higher sensitivity for PFC (Whittington et al. 2000a, Sergeant et al. 2002) for flock testing than in Verdugo et al. (2014a), which was the parent study in our case. Multiple factors may be attributable to differences in PFC sensitivity observed between these overseas studies and the parent study. Some examples include the difference in pool size and or the number of pools sampled per flock (both higher in overseas studies) and possible differences in contamination rate between the two different labs or the distribution of the multi- or paucibacillary cases in sampled flocks (Whittington et al. 2000a). Hence caution is required for interpretation of the lower sensitivity of the PFC presented here in more general terms.

Ideally, the accurate diagnosis of exposure to *Map* or the pathogen's presence in a cattle herd is determined by testing all adult animals using a combination of at least two tests in parallel in addition to environmental sampling (Correa-Valencia et al. 2017). Such an approach may neither be biologically warranted, economically feasible nor logistically practical in large-scale ovine farming systems. However, conclusive evidence about the utility of various test combinations to determine a flock's infection and shedding status is still lacking. Hence, more research is warranted.

Individual faecal samples from flocks with predetermined PFC status were tested by qPCR in the present study. However, neither the original pooled faecal samples nor information about their time-to-culture (as a proxy for quantification as faecal samples with a higher number of *Map* cells are more likely to take a relatively less time to culture as shown by a growth index) was available. In addition to assessing the relationship between the qPCR results and the ELISA sample to positive (S/P) ratio as presented in the present study, it would have been interesting to include the time-to-culture data and examine the correlation between the three factors instead of two as currently presented. Additionally, a parallel comparison between PFC and qPCR on pools of fresh faecal samples as tools to define flock status would have been worthwhile. Nevertheless, as the original pooled faecal samples for PCR purposes was not available and re-pooling these samples would not have been the same as the original pool, we did not compare these two methods in our study.

The serum samples tested by ELISA were tested at two different times with a gap of approximately eight years of freeze preservation at -80°C between them. While individual serum samples from PFC negative pools were tested in 2012 at the end of the survey by Verdugo (2013), the serum samples from the positive pools were tested in July 2018. In general, frozen serum is considered a stable specimen (Cuhadar et al. 2013), and in our case serum samples belonging to both the PFC negative and positive pools were tested in the same laboratory with similar ELISA kits albeit at different times. Hence, we assumed no effect of time on ELISA results in these two categories of serum samples. We believe this assumption was reasonable.

## 6.5 Opportunities and challenges in genomic analyses of *Map* genomes

The first complete *Map* genome was published in 2005 (Li et al. 2005). At the time of writing this thesis (Jan 2019), 11 complete *Map* genomes were publicly available in the National Center for Biotechnology Information (NCBI) database<sup>2</sup>. The advent of next generation sequencing, which is a catch-all term used to denote the non-Sanger-based high-throughput DNA sequencing technologies, has dramatically reduced both the cost and time required to sequence a bacterial genome. Consequently, whole genome sequencing has now become a standard procedure in many labs around the world for comparative bacterial genomics. This unprecedented growth in NGS data has led to a cost shift from data acquisition to data assembly, analysis, and management (Land et al. 2015).

The ability to examine full genomes and capture a relatively broader spectrum of genetic variation compared to Sanger sequencing are some advantages of NGS (Behjati & Tarpey 2013). The general approach to analysis of NGS data for detecting genetic differences, which range from single nucleotide variants (SNV) to genetic insertions or deletions (indels) or rearrangements, requires the use of a reference genome to compare the study samples. Such a reference can be obtained from openly available sources such as NCBI's database or created using a *de novo* assembly approach if a suitable genome does not

<sup>2</sup><https://www.ncbi.nlm.nih.gov/>

currently exist or is not easily available. Short read-length, repetitive regions, polymorphisms, missing data and sequencing errors create challenges for the *de novo* assembly approach (Lischer & Shimizu 2017).

Given the nucleotide-level resolution attainable from NGS, which has now progressed beyond mere DNA sequence analysis, it will continue to become the major tool for comparative bacterial genomics (Quainoo et al. 2017). However, some intrinsic challenges remain unmitigated; for instance, a considerable bioinformatics expertise is required for NGS data analysis workflows, and the appropriate selection of analytical software tools from a myriad of open-source and proprietary sources is often difficult (Kulkarni & Frommolt 2017). This situation is complicated by species uniqueness of bacterial genomes making it impossible to identify a single NGS data analysis strategy that suits every species of bacterial pathogen.

At the time this project was undertaken and raw data from WGS of *Map* isolates (described in Chapter 5 of this thesis) received, a closed Type S1 *Map* genome for use as a reference was not available. Due to approximately 3,000 single nucleotide polymorphism SNP differences between sheep type isolates in our study and K-10, a cattle type reference strain from the USA (complete genome available at NCBI, reference sequence number NC\_002944.2), which was the first closed *Map* genome that was publicly available, the latter was deemed unsuitable for using as a reference genome to compare the New Zealand sheep isolates reported in our study.

In this present study, we did not obtain an acceptable quality *Map de novo* genome assembly of the paired Illumina reads for any of the field isolates. *Map* genomes feature the presence of insertion sequences (IS element), an approximately 1400 nucleotide base sequence (Semret et al. 2006) in multiple copies (in some cases as many as 17 or more copies per genome) and a high GC content of around 69% (Li et al. 2005). The high GC content (Chen et al. 2013) in *Map* genomes and the inability of the short Illumina reads, with a maximum length of 250 base pairs, to span the entire length of the IS elements, might have affected the *de novo* assembly in our case. For *in silico* analysis, repeat sequences such as IS elements are a source of ambiguities in alignment and assembly which can lead to biased and error-prone results (Treangen & Salzberg 2012). Hence, for this analysis, we completely sequenced a Type S1 genome of an Australian strain, Telford 9.2 (Brauning et al. (2019), NCBI reference sequence: CP033688) using PacBio technology

which after further processing was used as a reference genome against which genomes of the New Zealand-Australian isolates in our study were mapped for SNV analyses.

Results presented in Chapter five of this thesis are descriptive results of SNVs identified in 69 Type S1 isolates. Most of the analyses was based on results generated using Snippy, an open-source SNV detection pipeline. Of all of the SNVs detected by Snippy-core analysis, 481 of the 672 (71.5%) were detected in only one genome and therefore were of less use for making phylogenetic comparisons, since these may have not been subjected to the same amount of selective pressures as SNVs detected in more than one isolate. In agreement with the findings of other groups, (Bryant et al. 2016) these results reflect the slow tendency of new changes to be incorporated and fixed into *Map* populations.

This group of isolates was from sheep from a variety of regions in the North and South Island of New Zealand and also a representative of a type that is predominant in Australia. This group were found to be identical or nearly identical to one another by traditional typing methods. While variable number of tandem repeats-short sequence repeat (VNTR-SSR) profiles were known for 65 out of 69 isolates, we selected an additional nine isolates for strain typing by RFLP IS900 and confirmed they were all Type S1. The latter list included isolate ags36, which had the highest number of SNVs compared to the reference. Based on the RFLP IS900 status of this subset of isolates and the genotypic closeness among these isolates by SNV analyses, we have assumed that all isolates in this study were likely to be Type S1.

With the extra resolution provided by analysis at the whole genome level, more differences were detected. Although these isolates differed from one another by 0–164 (median 40) SNVs, when we compared them to Type S1/S3 isolates from a different study (Bryant et al. 2016) we found that the New Zealand isolates were a genetically distinct group that differed from European Type S1 by hundreds and from Type S3 by over a thousand of SNVs. This survey illustrates the uniqueness of the type that predominates in New Zealand sheep flocks.

Several explanatory variables were assumed to be phenotypic measures of *Map* infection in the present study but, perhaps due to the slow rate at which *Map* changes, most were not positively associated with *Map* genotype. It is acknowledged that in the present study it was not possible to consider for additional factors such as host genomics, time post infection or infection dose that could also have affected the apparent lack of association

between *Map* genotype and phenotype of *Map* infection. Our preliminary results indicate that although there is a significant correlation between genotype and physical location of the *Map* host, differences in terms of how *Map* infection progresses and impacts on the ultimate disease outcome in an individual sheep might be affected by factors other than genotypically determined difference in pathogenicity, such as infectious dose, host immune response, host age, nutritional state and production stress. SNVs identified in the present study did not belong to a particular gene or gene family. However, we intend to use these data for further analysis for gene ontology enrichment analysis, which will allow us to identify over-represented genes that might have association with phenotypic expression of PTb as well as patterns of selection on protein genes based on the ratio of the non-synonymous to synonymous substitutions. Such analysis will provide better insights into the evolutionary biology of Type S *Map* isoaltes in New Zealand context.

## 6.6 Conclusions

When this PhD was undertaken, the aim was to understand ovine PTb in a more comprehensive manner than reported previously. The review of current PTb control programs in New Zealand for the cattle, sheep and deer industries (chapter 2) was the first of its kind and has now been published in a peer-reviewed journal (Gautam et al. 2018b). While PTb is endemic at flock or herd level in all major ruminant livestock farms in New Zealand, no formal PTb control program exists for sheep or other livestock industries. Annual ewe mortality due to all reasons and ewe mortality attributable to clinical PTb in fine wool and other breeds were determined for the first time using longitudinal data collected from clinically affected commercial farms (chapter 3). Annual ewe mortality was similar in fine-wool and other breeds, but mortality in ewes as a result of clinical PTb was 2.7 times as high in fine-wool breeds than other breeds. These estimates of annual mortalities would be useful for modelling dynamics and/or economics of PTb in New Zealand commercial sheep flocks (for example (Marquetoux 2017)). Stochastic analysis showed if annual ewe mortality due to OJD is  $>1\%$ , vaccination in replacement lambs would be economically attractive. Until now the IFP estimates of *Map* infection were not available, although a previous survey (Verdugo et al. 2014a) reported that at least 70% of commercial sheep flocks in New Zealand were infected. In chapter 4 of this thesis, the median

IFP of *Map* shedding and antibody ELISA positivity in non-clinical commercial sheep flocks was estimated to be 13.5% and 10% respectively based on faecal qPCR and serum ELISA tests of individual samples from flocks with a predetermined PFC status. Approximately 1% ewes in qPCR positive flocks were supershedders. Finally, genotype of Type S *Map* isoalters from New Zealand sheep were determined for the first time using WGS data and a closed Type S genome as a reference (chapter 5). In concordance with results of a previous study that used traditional genotyping methods (Verdugo et al. 2014b), the Type S *Map* genotypes from New Zealand sheep were similar across the country and not affected by the type of breed or disease outcome in hosts.

### 6.6.1 Further work area

Based on our results and data generated in this research we have identified several areas and hypotheses related to ovine PTb that could inform and benefit further studies in future. Given that JDRC is now formally closed, acquiring funding support from the ovine industry or generic government science research funds (for example Marsden fund) will probably be the most important challenge for such future studies.

A national scale case-control study to investigate the overall efficiency and cost-benefit analysis of participating in the voluntary PTb control program is currently recommended for New Zealand dairy cattle and deer industries. As shown in chapter 3 of this thesis, most fine wool ovine flocks with clinical PTb incidence of >1% per year would benefit from vaccination as a control intervention. Currently there are not any formal PTb control programs recommended for the New Zealand sheep industry. A comparative study to investigate temporal changes between prevalence of *Map* infection in infected farms with and without vaccination and/or other clinical PTb targeted strategies will be helpful to determine the change in IFP over time and assess the risks better. Such a study will also provide a better understanding of the prevalence cut point for intervention in New Zealand sheep flocks.

Based on results presented in this thesis (chapter 3), fine wool breed flocks were likely to be affected by clinical PTb and were more likely to have higher economic returns from vaccination. Hence a simulation study to estimate the economic cost of ovine PTb and the cost-benefit of vaccination could be targeted for fine wool flocks. Such a study should

take into account both clinical and sub-clinical effects of PTb and potential trade impact such as trade restrictions at international levels. Parameters, such as PTb incidence and prevalence and other production data required for this study should preferably be generated longitudinally. The sub-clinical effects of ovine PTb are not adequately established and quantified for New Zealand. Hence a prerequisite for such a simulation study would be to evaluate and obtain robust estimates of these sub-clinical effects. With respect to the potential trade impact due to ovine PTb, the first thing that needs to be addressed is to examine the risk of transmission or exposure to people from sheep meat.

Several studies have reported presence of *Map* cells in raw meat tissues [for example Redacliff et al. (2010), Pribylova et al. (2011), Savi et al. (2015)]. A previous New Zealand study in which the presence of *Map* in sheep meat was reported (Smith et al. 2011) was limited to ewes that were either clinically affected or in direct contact with the clinical ewes prior to slaughter. A study of random meat samples across multiple farms in the country would provide a more robust overall prevalence estimate of infection in meat as well as between-farm variations in prevalence. The next target could then be investigation of factors that contribute to these variations, potentially a case control study. Finally, results of these studies could then be used in assessing and or/managing the potential trade impact due to ovine PTb.

Based on qPCR study results presented in this thesis (chapter 4) it was not possible to infer the survival of *Map* cells in the long-term frozen faecal samples. To the best of our knowledge the effect of long-term storage of faecal and serum samples at -80°C on PTb diagnostics has also not been reported elsewhere and might be an area for further research. These results from such studies might be helpful in cases where immediate testing of faecal or serum samples collected will not be possible or data from different times need to be compared.

Based on SNV analyses of WGS data, we did not find evidence of virulence difference among New Zealand ovine *Map* isolates. Experimental infection models including hosts with controlled genotype that are susceptible to clinical PTb (for instance Merino) with a large range of disease outcome (for example as those reviewed in Whittington et al. (2017)) might help to draw stronger inference in support of or against the virulence hypothesis. Alternative hypotheses such as the interplay between the host and the pathogen factors in development of clinical PTb should also be investigated or methods other than

WGS for data analysis be used.

Differentiation of passive transfer of *Map* pathogens and active *Map* shedding by defining the respective cut off points for PCR based assays and the potential role of PCR as a diagnostic tool for making on farm cull decisions in sheep flocks might be an area for further research. Likewise, a more methodical evaluation of pooling for qPCR as a predictor of flock status would be determined by testing pools of fresh faecal samples by qPCR and using culture as the gold standard.



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**R codes used for estimation of ewe mortality  
and benefit-cost analysis of vaccination in a  
hypothetical flock of 2,000 breeding ewes  
clinically affected with paratuberculosis in New  
Zealand**

---

```
#mort = mortality
#FW = fine wool breeds
#OT = other breeds

rm(list=ls()) # Clear R console
library(triangle)

#Overall mortality
mortFW<- 0.076
mortOT<- 0.076

#OJD based on farmers farmers' observations
susFW<- 0.36
susOT<- 0.211

#OJD based on necropsy
pmFW<-0.6813
pmOT<-0.2698

mortojdFW<-mortFW*susFW*pmFW
mortojdFW
mortojdOT<-mortOT*susOT*pmOT
mortojdOT

#Repeat simulation based on 10,000 draws
n <- 10000
mortFW<-numeric(n)
mortOT<-numeric(n)

susFW<-numeric(n)
susOT<-numeric(n)
```

**Figure A.1**

```
pmFW<-numeric(n)
pmOT<-numeric(n)

mortojdFW<-numeric(n)
mortojdOT<-numeric(n)
mortratio<-numeric(n)
bcratio<-numeric(n)

#Define flock size
fs<-2000

#Ewe death per year
deweFW<-numeric(n)
deweOT<-numeric(n)

#Ewe carcass value (dollars)
ewecvalueFW<-50
ewecvalueOT<-70

#Ewe carcass loss
ecarlossFW<-numeric(n)
ecarlossOT<-numeric(n)

#Ewe fleece values (dollars)
ewefvalueFW<-40
ewefvalueOT<-17

#Total wool loss
twoollossFW<-numeric(n)
twoollossOT<-numeric(n)
```

Figure A.2

```
#Total ewe loss
tewelossFW<-numeric(n)
tewelossOT<-numeric(n)

#Tailing percentage
lamb_pFW<-1.01
lamb_pOT<-1.20
lamb_pFW<- numeric(n)
lamb_pOT<- numeric(n)

#Lamb loss
lamblossFW <- numeric(n)
lamblossOT <- numeric(n)

#Lamb carcass values (dollars) and lamb wool loss
lamb_carcassvFW<-90
lamb_carcassvOT<-90
lambclossFW<-numeric(n)
lambclossOT<-numeric(n)

#Lamb fleece values and wool loss
flv_FW<-40
flv_OT<-0
lambwlossFW<-numeric(n)
lambwlossOT<-numeric(n)

#Gross lamb loss
glamblossFW<-numeric(n)
glamblossOT<-numeric(n)
```

**Figure A.3**

```
#Percentage of lambs delayed for finishing
delay_finish_IFW<-0.5
delay_finish_IOT<-0.5
delay_finish_IFW<-numeric(n)
delay_finish_IOT<-numeric(n)

#Days to finish post weaning
day_finish_pw_FW<-240
day_finish_pw_OT<-100
day_finish_pw_FW<- numeric(n)
day_finish_pw_OT<- numeric(n)

#Death rate weaning to finishing
death_wean_fin_FW<-0.03
death_wean_fin_OT<-0.025
death_wean_fin_FW<-numeric(n)
death_wean_fin_OT<-numeric(n)

#Total extra lamb days saved
te_lday_sav_FW<-numeric(n)
te_lday_sav_OT<-numeric(n)

#Health measures cost per day
hmeasure_FW<-0.02
hmeasure_OT<-0.02

#Feed cost per day
f_cost_FW<-0.09 # feed cost per day as a fixed value
f_cost_OT<-0.09

#Total lamb cost saved
```

Figure A.4

```
tl_cost_sav_FW<- numeric(n)
tl_cost_sav_OT<- numeric(n)

#Total lamb loss
tlamb_loss_FW<-numeric(n)
tlamb_loss_OT<-numeric(n)

#Annual replacement rates
arep_rate_FW<-0.25
arep_rate_OT<-0.25
arep_rate_FW<-numeric(n)
arep_rate_OT<-numeric(n)

#Replacment per ewe (dollars)
vreplace_FW<-105
vreplace_OT<-105

#Total costs of replacements
treplace_cost_FW<-numeric(n)
treplace_cost_OT<-numeric(n)

#Total costs of ewe death
tcost_eddeath_FW<-numeric(n)
tcost_eddeath_OT<-numeric(n)

#Proportion of suspect live ewes in low body condition that tested ELISA postive
ps_elisapos_FW<-0.39
ps_elisapos_OT<-0.09
ps_elisapos_FW<-numeric(n)
ps_elisapos_OT<-numeric(n)
```

**Figure A.5**

```
#Proportion of ewes with low body condition scores.
```

```
ps_lbcs_FW<-0.05
```

```
ps_lbcs_OT<-0.05
```

```
ps_lbcs_FW<-numeric(n)
```

```
ps_lbcs_OT<-numeric(n)
```

```
#Profit per ewe per year
```

```
p_pewe_yr_FW<-45
```

```
p_pewe_yr_OT<-35
```

```
p_pewe_yr_FW<-numeric(n)
```

```
p_pewe_yr_OT<-numeric(n)
```

```
#Productive years lost to OJD
```

```
prod_yloss_FW<-0.48
```

```
p_pewe_yr_OT<-0.48
```

```
prod_yloss_FW<-numeric(n)
```

```
prod_yloss_OT<-numeric(n)
```

```
#Preclinical loss per ewe
```

```
sloss_pewe_FW<-numeric(n)
```

```
sloss_pewe_OT<-numeric(n)
```

```
sloss_ojd_FW<-numeric(n)
```

```
sloss_ojd_OT<-numeric(n)
```

```
# Total production cost
```

```
tprod_cost_FW<-numeric(n)
```

```
tprod_cost_OT<-numeric(n)
```

```
#Percent lamb loss
```

```
p_lloss_FW<-numeric(n)
```

```
p_lloss_OT<-numeric(n)
```

Figure A.6

```
#Percent ewe loss
p_ewloss_FW<-numeric(n)
p_ewloss_OT<-numeric(n)

#Percent replacement cost
p_rcost_FW<-numeric(n)
p_rcost_OT<-numeric(n)

#Percent subclinical loss
p_scloss_FW<-numeric(n)
p_scloss_OT<-numeric(n)

#Loss per ewe
l_pewe_FW<-numeric(n)
l_pewe_OT<-numeric(n)

#Percentage extra lambs kept for replacment
elambs_replace_FW<-0.1
elambs_replace_OT<-0.1

#Percentage of ewe lambs vaccinated
pelambs_vacc_FW<-0.60
pelambs_vacc_OT<-0.50
n_elamb_vacc_FW<-numeric(n) # no of ewe lambs vaccinated as a calculated value
n_elamb_vacc_OT<-numeric(n)

#Gudair vaccine cost (vaccine + labour cost)
vac_cost_FW<-4
vac_cost_OT<-4
#Total lamb vaccine cost
```

Figure A.7

```
tl_vcost_FW<-numeric(n)
tl_vcost_OT<-numeric(n)

#Vaccine efficacy
vacc_eff_FW<- 0.9
vacc_eff_OT<- 0.9

#Achievable benefit from vaccination
ach_ben_FW<- numeric(n)
ach_ben_OT<- numeric(n)

#Cost benefit of vaccination used as a calculated value
cb_vacc_FW<- numeric(n)
cb_vacc_OT<- numeric(n)

#Benefit cost ratio of vaccination
bcr_FW<- numeric(n)
bcr_OT<- numeric(n)

#START SIMULATION
for (i in 1:n) {
# Fine wool breeds
mortFW[i]<-rbeta(1, 8.717, 94.8221)

susFW[i]<- rbeta(1, 3.9543, 6.2522)

pmFW[i]<- rbeta(1, 4.9044, 2.711)

mortojdFW[i]<- mortFW[i]*susFW[i]*pmFW[i]
```

Figure A.8

```

deweFW[i]<- mortojdFW[i]*fs
ecarlossFW[i]<-deweFW[i]*ewecvalueFW
twoollossFW[i]<-deweFW[i]*ewefvalueFW
tewelossFW[i]<-ecarlossFW[i]+twoollossFW[i]

lamb_pFW[i]<- rnorm(1, 1.0, 0.17)
lamblossFW[i] <- deweFW[i] * lamb_pFW[i]

lambclossFW[i] <- deweFW[i] * lamb_pFW[i] *lamb_carcassvFW
lambwlossFW[i]<- deweFW[i] * lamb_pFW[i] * flv_FW
glamblossFW[i]<- lambclossFW[i]+lambwlossFW[i]

delay_finish_IFW[i]<- rnorm(1, 0.5, 0.05)
day_finish_pw_FW[i]<- rnorm(1,240,24)
death_wean_fin_FW[i]<-rbeta(1,6.4243,176.3867)
te_lday_sav_FW[i]<- lamblossFW[i]*delay_finish_IFW[i]*day_finish_pw_FW[i]*(1-
death_wean_fin_FW[i])

tl_cost_sav_FW[i]<- te_lday_sav_FW[i]*(hmeasure_FW+f_cost_FW)
tlamb_loss_FW[i]<- glamblossFW[i]-tl_cost_sav_FW[i]

arep_rate_FW[i]<- rbeta(1, 3.8761, 9.6284)
treplace_cost_FW[i]<- deweFW[i]*vreplace_FW

tcost_eddeath_FW[i]<-tewelossFW[i]+tlamb_loss_FW[i]+ treplace_cost_FW[i]

ps_elisapos_FW[i]<- rbeta(1,3.0818,3.1668)
ps_lbcv_FW[i]<- rbeta(1,6.1946,99.6983)
p_pewe_yr_FW[i]<- rnorm(1,45,4.5)
prod_yloss_FW[i]<- rnorm(1,0.6,0.06)

```

Figure A.9

```

sloss_pewe_FW[i]<- ps_elisapos_FW[i]*ps_lbc FW[i]*p_pewe_yr_FW[i]*prod_yloss_FW[i]
sloss_ojd_FW[i]<- sloss_pewe_FW[i]*fs
tprod_cost_FW[i]<- tcost_eddeath_FW[i]+ sloss_ojd_FW[i]

p_lloss_FW[i]<- tlamb_loss_FW[i]/tprod_cost_FW[i]
p_e loss_FW[i]<- tewelossFW[i]/tprod_cost_FW[i]
p_rcost_FW[i]<- treplace_cost_FW[i]/tprod_cost_FW[i]
p_scloss_FW[i]<- sloss_ojd_FW[i]/tprod_cost_FW[i]
l_pewe_FW[i]<- tprod_cost_FW[i]/fs

n_elamb_vacc_FW[i]<- (fs*pelambs_vacc_FW)/2
tl_vcost_FW[i]<- n_elamb_vacc_FW[i]*vac_cost_FW

vacc_eff_FW[i]<-rbeta(1,5.3842,1.4871)

ach_ben_FW[i]<- tprod_cost_FW[i]*vacc_eff_FW[i]
cb_vacc_FW[i]<-ach_ben_FW[i]-tl_vcost_FW[i]
bcr_FW[i]<- ach_ben_FW[i]/tl_vcost_FW[i]

# Other breeds

mortOT[i]<- rbeta(1, 10.2378, 113.3121)
susOT[i]<- rbeta(1, 1.4972, 2.8591)
pmOT[i]<- rbeta(1, 3.3551 , 7.1501)

mortojdOT[i]<- mortOT[i]*susOT[i]*pmOT[i]

deweOT[i]<- mortojdOT[i]*fs
ecarlossOT[i]<- deweOT[i]*ewecvalueOT
twoollossOT[i]<- deweOT[i]*ewefvalueOT

```

Figure A.10

```

tewelossOT[i]<- ecarlossOT[i]+twoollossOT[i]

lamb_pOT[i]<- rnorm(1, 1.3, 0.05)
lamblossOT[i] <- deweOT[i] * lamb_pOT[i]

lambclossOT[i]<- deweOT[i] * lamb_pOT[i] *lamb_carcassvOT
lambwlossOT[i]<- deweOT[i] * lamb_pOT[i] * flv_OT
glamblossOT[i]<- lambclossOT[i]+lambwlossOT[i]

delay_finish_IOT[i]<- rnorm(1, 0.5, 0.05)
day_finish_pw_OT[i]<- rnorm(1,100,10)
death_wean_fin_OT[i]<- rbeta(1, 6.4817, 214.7868)
te_lday_sav_OT[i]<- lamblossOT[i]*delay_finish_IOT[i]*day_finish_pw_OT[i]*(1-
death_wean_fin_OT[i])

tl_cost_sav_OT[i]<- te_lday_sav_OT[i]*(hmeasure_OT+f_cost_OT)
tlamb_loss_OT[i]<- glamblossOT[i]-tl_cost_sav_OT[i]

arep_rate_OT[i]<- rbeta(1, 3.8761, 9.6284)
treplace_cost_OT[i]<- deweOT[i]*vreplace_OT

tcost_eddeath_OT[i]<- tewelossOT[i]+tlamb_loss_OT[i]+ treplace_cost_OT[i]
ps_elisapos_OT[i]<- rbeta(1, 6.1946, 99.6983)

ps_lbcS_OT[i]<- rbeta(1,6.1946,99.6983)
p_pewe_yr_OT[i]<- rnorm(1,35,3.5)
prod_yloss_OT[i]<- rnorm(1,0.6,0.06)
sloss_pewe_OT[i]<- ps_elisapos_OT[i]* ps_lbcS_OT[i]*p_pewe_yr_OT[i]*prod_yloss_OT[i]
sloss_ojd_OT[i]<- sloss_pewe_OT[i]*fs
tprod_cost_OT[i]<- tcost_eddeath_OT[i]+ sloss_ojd_OT[i]

```

Figure A.11

```
p_lloss_OT[i]<- tlamb_loss_OT[i]/tprod_cost_OT[i]
p_ellos_OT[i]<- tewelossOT[i]/tprod_cost_OT[i]
p_rcost_OT[i]<- treplace_cost_OT[i]/tprod_cost_OT[i]
p_scloss_OT[i]<- sloss_ojd_OT[i]/tprod_cost_OT[i]
l_pewe_OT[i]<- tprod_cost_OT[i]/fs

n_elamb_vacc_OT[i]<- (fs*pelamb_vacc_OT)/2
tl_vcost_OT[i]<- n_elamb_vacc_OT[i]*vac_cost_OT

vacc_eff_FW[i]<-rbeta(1,5.3842,1.4871)
vacc_eff_OT[i]<-rbeta(1,5.3842,1.4871)
ach_ben_OT[i]<- tprod_cost_OT[i]*vacc_eff_OT[i]
cb_vacc_OT[i]<- ach_ben_OT[i]-tl_vcost_OT[i]
bcr_OT[i]<- ach_ben_OT[i]/tl_vcost_OT[i]

mortratio[i]<- mortojdFW[i]/mortojdOT[i]
bcratio[i]<-bcr_FW[i]/bcr_OT[i]

}

summary(bcr_FW)
summary(bcr_OT)

summary(mortojdFW)
summary(mortojdOT)

# Comparison between OJD specific mortalities in FW and OT breeds
summary(mortojdFW/mortojdOT)
```

Figure A.12

```

#OJD mortality FW
summary(mortojdFW)
par(mfrow=c(1,2))
plot(density(round(mortojdFW*100, digits=4)),main="", lty=4,col="blue",
      cex.main=1, font.main=2, cex=3, cex.lab=1, xlab="annual OJD mortality (%)", ylab="", axes=TRUE,
      yaxt="n", xlim=c(0,10) ) #, ylim=c(0,50) ,xaxt="n", yaxt="n"
legend(x="topright", 0.09, legend=c(paste("Median 1.8% "),paste("IQR 1.2 - 2.7%")), cex=.75)
abline(v=1.8,col="red",lty=1)

summary(mortojdOT)
plot(density(round(mortojdOT*100, digits=4)),main="", lty=4,col="blue",
      cex.main=1, font.main=2, cex=3, cex.lab=1, xlab="annual OJD mortality (%)", ylab="", axes=TRUE,
      yaxt="n",xlim=c(0,10)) #, ylim=c(0,4) ,xaxt="n"
legend(x="topright", 0.09, legend=c(paste("Median 0.69%"),paste("IQR 0.3 - 1.2%")), cex=.75)
abline(v=0.68,col="red",lty=1)

summary(bcr_FW)
plot(density(bcr_FW),main= "", lty=4,col="blue",
      cex.main=1, font.main=2, cex=3, cex.lab=1, xlab="benefit:cost ratio", ylab="", axes=TRUE,
      yaxt="n",xlim=c(0,12)) #, ylim=c(-1,4) ,xaxt="n"
legend(x="topright", 0.09, legend=c(paste("Median 4.1"),paste("IQR 2.6-6.0")), cex=.75)
abline(v=1, col="red",lty=4)

summary(bcr_OT)
plot(density(bcr_OT),main="Benefit/cost ratio of vaccination (other breeds)", lty=4,col="blue",
      cex.main=1, font.main=2, cex=3, cex.lab=1, xlab="benefit:cost (dollars)", ylab="", axes=TRUE,
      yaxt="n",xlim=c(0,10)) #, ylim=c(-1,4) ,xaxt="n"
legend(x="topright", 0.09, legend=c(paste("Median 1.6"),paste("IQR 0.8 - 3.0")), cex=.75)

```

Figure A.13

```
abline(v=1, col="red",lty=4)
c(quantile(bcr_OT, 0.33), quantile(bcr_OT, 0.9))

# points(density(bcr_OT), col="green", pch=1, lty=3)
par(mfrow=c(1,2))

dev.off()

#plot the relationship between OJDmortality and BCR
plot(mortojdFW*100,bcr_FW, xlim=c(0,4) , ylim=c(0,5), xlab="annual mortality due to OJD (%)",
ylab="benefit-cost ratio of vaccination", cex=0.1)

abline(v=1, col="black",lty=2)
abline(h=1, col="black",lty=2)

plot(mortojdOT*100, bcr_OT, xlim=c(0,4), ylim=c(0,5), xlab="annual mortality due to OJD (%)",
ylab="benefit-cost ratio of vaccination", cex=0.1)

abline(v=1, col="black",lty=2)
abline(h=1, col="black",lty=2)
```

Figure A.14

---

# Complete genome sequence of the Telford Type S strain of *Mycobacterium avium* subsp. *paratuberculosis*

---

**Abstract** – *Mycobacterium avium* subsp. *paratuberculosis* is the causative agent of Johne’s disease (JD). Here, we report the complete genome sequence of Telford 9.2, a well-characterized representative strain of the *M. avium* subsp. *paratuberculosis* S subtype that is endemic in New Zealand (NZ) and Australian sheep.

Brauning R, Plain K, Gautam M, Russel T, Correa C.C, Biggs P, Whittington R, Murray A, Price-Carter M (2019) Complete genome sequence of the Telford type S strain of *Mycobacterium avium* subsp. *paratuberculosis* *Microbiology Resource Announcement*. (Accepted for publication Feb 2019 ). doi:10.1128/MRA.00004-19

*Mycobacterium avium* subsp. *paratuberculosis* is the causative agent of Johne’s disease (JD), a chronic, generally subclinical but sometimes fatal granulomatous enteritis of ruminants (Garcia & Shalloo 2015). *M. avium* subsp. *paratuberculosis* Type S (also called either subtype I or subtype III) has primarily been isolated from sheep but also from other ruminant species (reviewed in (Stevenson 2015)). Only draft genomes (Wynne et al. 2011, Bannantine et al. 2012, Möbius et al. 2015) of *M. avium* subsp. *paratuberculosis* Type S are currently available. Here, we announce the complete genome sequence of Telford 9.2, an IS1311 Type S IS900 restriction fragment length polymorphism (RFLP) Type S1 strain. This is a clonal culture (passage level 5, including its primary isolation from sheep feces) of an isolate from a clinically infected sheep from New South Wales, Australia. It has been used as inoculum in an experimental model for clinical JD in sheep (Begg

et al. 2010, Dukkipati et al. 2016), characterized genetically (Marsh et al. 2006), and is representative of the *M. avium* subsp. *paratuberculosis* type endemic in Australian and New Zealand (NZ) sheep (Whittington et al. 2000c, Verdugo et al. 2014b, Gautam et al. 2018b).

For Illumina and PacBio sequencing, bacterial stock was inoculated into either supplemented Middlebrook 7H9 (Whittington 2010) (Illumina) or M7H9C (PacBio) (Whittington et al. 2013) liquid medium, cultured for 3 to 4 weeks, and then cultivated on modified Middlebrook 7H10 solid medium (Whittington 2010), harvested, and stored at -80°C.

Genomic DNA was prepared for both PacBio and Illumina sequencing by isopropanol precipitation and 70% ethanol wash of cetyltrimethylammonium bromide (CTAB)/ phenol-chloroform-extracted cellular material after stepwise enzymatic digestion with lysozyme, RNase A, and proteinase K. For PacBio sequencing, the DNA was also digested with mutanolysin prior to proteinase K digestion and subjected to extra cleanup and concentration on Ampure PB magnetic beads.

A PacBio library was constructed and sequenced at the Ramaciotti Centre in Sydney, Australia, using P6-C4 chemistry, and sequenced to a coverage depth of 80X on the PacBio RS II platform on a single-molecule real-time (SMRT) cell. It was improved with Illumina MiSeq 250-bp paired-end (PE) reads generated by sequencing two cultures of the Telford 9.2 reference strain. MiSeq-indexed libraries were created at New Zealand Genomics Limited using Nextera XT DNA kits (Illumina, San Diego, CA). Average coverage was 120X from PacBio data and 135X from Illumina data. There were 2.5 million Illumina PE reads (909 Mbp) and 150,000 PacBio reads prefilter (555 Mbp; N50 value, 10.5 kbp). PacBio reads went through default filtering steps in SMRTPipe v1.87.139483, which reduced read numbers to 63,000 (491 Mbp; N50 value, 10.8 kbp), and were assembled using PacBio Hierarchical Genome Assembly Process v3 (HGAP3; SMRT analysis v2.3.0) into a single contig (Telford1) of the size expected for a complete *M. avium* subsp. *paratuberculosis* genome and with a GC content of 69.2%, which is typical of *M. avium* subsp. *paratuberculosis* (Li et al. 2005, Wynne et al. 2011, Bannantine et al. 2012, Möbius et al. 2015). The PacBio-based assembly was improved by removing a 9-kbp overlap between the start and the end of the genome, orienting the genome with the start position at the beginning of the *dnaA* gene and mapping Illumina reads onto the PacBio assembly to detect and repair small-scale variations, as described in Table B.1.

Telford1 has a sequence length of 4,907,428 bases, 4,377 coding sequences as predicted with the NCBI Prokaryotic Genome Annotation Pipeline (NCBI 2013), and an *in silico* IS1311 Type S IS900 RFLP Type S1 (Price-Carter & Whittington 2019).

**Data availability.** The genome assembly is available at GenBank under accession number CP033688 and the BioProject accession number PRJNA504678; raw data are available under SRA accession numbers SRX4997502 (Illumina) and SRX4997501 (PacBio).

Table B.1: Discrepancies between Illumina and PacBio data<sup>a</sup>

Position before fix	Variant type	Accepted solution	Pacbio allele	Illumina allele	Applied fix
780880 <sup>b</sup>	Indel	Illumina	T	TG	Insertion
931746	SNP	PacBio	C	G	na
1112469	Indel	Illumina	GCCCCC	GCCCCCC	Insertion
1302183	Indel	Illumina	AGGGG	AGGGGG	Insertion
1969375	Indel	Illumina	GCCCCC	GCCCCCC	Insertion
2128150	Indel	Illumina	ACCCCC	ACCCCCC	Insertion
2276090	Indel	Illumina	CGGGGG	CGGGGGG	Insertion
2577759	SNP	PacBio	G	A	na
2635929	Indel	Illumina	GCCCC	GCCCCC	Insertion
2642118	SNP	PacBio	C	T	na
2705636	Indel	Illumina	GCCCCC	GCCCCCC	Insertion
3024648	Indel	Illumina	T	TC	Insertion
3201490	SNP	PacBio	C	G	na
3201602	SNP	PacBio	A	G	na
3211597	Indel	Illumina	CGGGGGGG	CGGGGGGGG	Insertion
3450836	Indel	PacBio	CATCGTCGCGCCGTG CTGGGCG- GCCAGCGC GTCGCCGACCAGGCT GCGCGCCGGCTCGAC GCGC- CGCGCGGCCCG CAGCGCCT- GCTGGG	C	na
4313098	SNP	Illumina	N	G	Base change
4314018	Indel	Illumina	GTTT	GTT	Deletion
4318473	Indel	Illumina	AC	A	Deletion
4319018	Indel	Illumina	AC	A	Deletion
4319236	Indel	Illumina	GTTT	GTT	Deletion
4319286	Indel	Illumina	CGGGG	CGGG	Deletion
4320148	Indel	Illumina	ACGCGCGC	ACGCGC	Deletion
4371898	SNP	PacBio	G	T	na
4416918	Indel	PacBio	CCGTTCCGGCGCCGAG CGTACGCCAGCGTG GCGCTCGGGGCCG GCGC- CACGCTGGCGT GACG	CCG	na
4421523	Indel	Illumina	GCCCC	GCCCCC	Insertion
4572001	SNP	PacBio	G	A	na
4594338	Indel	Illumina	ACCCC	ACCCCC	Insertion

<sup>a</sup>Illumina reads were mapped onto the PacBio assembly using BWA-MEM (Li & Durbin 2010) v0.7.17-r1188 with parameter “-M” and then variants (SNPs and indels) were detected (SAMtools (Li 2011) v1.3 with parameters “view -q 30 -F 256,” SAMtools v1.3 with parameters “mpileup -t DP, AD,” BCFtools v0.1.16 with parameters “call -cv”, BCFtools v0.1.16 with parameters “view -M2”). For each variant, a read depth greater than 10 was required, and a visual check of mapq values as well as the reference and alternative allele counts was performed. As a result of this analysis, for SNPs the PacBio alleles were accepted, for short indels the Illumina alleles were accepted, and for longer indels the PacBio alleles were accepted. All variants were verified by comparing 200 bp of flanking sequence (centered on the variants) to very closely related *Map* strains (Bannantine et al. 2012, Möbius et al. 2015) using the “map to a reference” function in Geneious (Kearse et al. 2012) and also comparing this fragment to *M. avium* subsp. *paratuberculosis* strains included in NCBI taxid 1770 using the NCBI BLAST service with default settings.

<sup>b</sup>For the indel at position 780,880, the Telford1 sequence differed from closely related strains in both PacBio and Illumina alleles; Sanger sequencing confirmed the Illumina call.

SNP= single nucleotide polymorphism; na= no action

**APPENDIX C**

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**Statement of contribution**

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### STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Milan Gautam
Name/title of Primary Supervisor:	Cord Heuer
Name of Research Output and full reference:	
Review Article. Gautam M, Ridler A, Wilson PR, Heuer H, (2018) Control of clinical paratuberculosis in New Zealand pastoral livestock. New Zealand Veterinary Journal 66 (1), 1 – 8. doi: 10.1080/00480169.2017.1379914	
In which Chapter is the Manuscript /Published work:	Chapter 2
Please indicate:	
<ul style="list-style-type: none"> <li>The percentage of the manuscript/Published Work that was contributed by the candidate:</li> </ul>	80 %
and	
<ul style="list-style-type: none"> <li>Describe the contribution that the candidate has made to the Manuscript/Published Work:</li> </ul>	
wrote the first draft of the manuscript, revised the manuscript based on reviewers' first comments submitted to the journal	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	
Date:	27 March 2019
Primary Supervisor's Signature:	
Date:	27-03-2019

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**Figure C.1**

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Name of candidate:	Milan Gautam
Name/title of Primary Supervisor:	Cord Heuer
Name of Research Output and full reference:	
Original article. Gautam M, Anderson P, Ridler A, Wilson P, Heuer H (2018) Economic cost of ovine Johne's disease in clinically affected New Zealand flocks and benefit-cost of vaccination. <i>Veterinary Sciences</i> 5(16), 1 – 13. doi:10.3390/vetsci5010016	
In which Chapter is the Manuscript /Published work:	Chapter 3
Please indicate:	
<ul style="list-style-type: none"> <li>The percentage of the manuscript/Published Work that was contributed by the candidate:</li> </ul>	80 %
and	
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analyzed data, wrote the first draft of the manuscript, revised the manuscript based on reviewers' first comments, submitted to the journal	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	
Date:	27 March 2019
Primary Supervisor's Signature:	
Date:	27-03-2019

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**Figure C.2**

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Name/title of Primary Supervisor:	Cord Heuer
Name of Research Output and full reference:	
Brauning R, Plain K, Gautam M, Russel T, Correa C.C, Biggs P, Whittington R, Murray A, Price-Carter M (2019) Complete genome sequence of the Telford type 5 strain of <i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> . <i>Microbiology Resource Announcement</i> . (Accepted for publication Feb 2019 ). doi:10.1128/MRA.00004-19	
In which Chapter is the Manuscript /Published work:	Appendix A
Please indicate:	
<ul style="list-style-type: none"> <li>The percentage of the manuscript/Published Work that was contributed by the candidate:</li> </ul>	35 %
and	
<ul style="list-style-type: none"> <li>Describe the contribution that the candidate has made to the Manuscript/Published Work:</li> </ul>	
Data analysis by comparing the pacbio sequence read and illumina reads of Telford 9.2 in NCBI's database. Wrote the first draft of the manuscript.	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	
Date:	27 March 2019
Primary Supervisor's Signature:	
Date:	27-03-2019

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**Figure C.3**